Hormonal response of *Acutodesmus obliquus* exposed to combined treatment with 24-epibrassinolide and lead

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Abstract

Phytohormones play key roles in many aspects of plant growth and development, as well as in resistance to abiotic stresses. The presence of heavy metal limits phytohormone endogenous level; however, the application of brassinosteroids (BRs) restores phytohormone homeostasis and reduces unfavorable consequences of heavy metal on plant growth. Therefore, the aim of this study was to (1) broaden previously published analyses/findings to study the effect of 24-epibrassinolide (EBL) and/or lead (Pb) on *Acutodesmus obliquus* phytohormone level after 5 days of cultivation using LC-QToF-MS quantification technique and (2) extend the total amount of quantified phytohormones in *A. obliquus*. The study confirmed that exogenous EBL increased the contents of gibberellin A₃ (GA₃), auxins (AXs) (indole-3-acetic acid, phenylacetic acid), BRs brassinolide, 24-epibrassinolide (EBL), 28-homobrassinolide, castasterone, 24-epicastasterone, typhasterol, cathasterone, 6-deoxotyphasterol, as well as different types of cytokinins (CKs): free bases, ribosides, and conjugates (*N*- and *O*-glucosides). On the other hand, treatment with Pb had an opposite effect on BRs, GA₃, AXs, and free bases, as well as ribosides of CKs. The abscisic acid (ABA) level decreased under EBL treatment but increased in response to Pb stress. The level of *N*-glucosides, as well as *O*-glucosides of CKs, also significantly increased in response to Pb alone. Interestingly, the co-application of EBL and Pb led to an increase in BRs, GA₃, AXs, and CKs content in the algae. Principal component analysis revealed that based on treatments, increase of GA₃, BRs, and AXs was found to be in the following order: 1 μM EBL > 0.01 μM Pb + 1 μM EBL > 500 μM Pb + 1 μM EBL > control > 0.01 μM Pb > 500 μM Pb. Moreover, very strong significant linear relations between almost all studied AXs and BRs were noted. Summarizing, this research did not only allow to detect the occurrence of ABA, GA₃, AXs, BRs, and CKs in *A. obliquus* for the first time but also to study the combined action of EBL and Pb, as well as compare it with treatment alone.

Keywords Auxins · Brassinosteroids · Cytokinins · Gibberellin · Green alga · Lead

Introduction

Plant hormones are naturally occurring organic compounds that play a significant role in most physiological processes of plants, such as growth and stress responses. These compounds mainly include brassinosteroids (BRs), auxins (AXs), cytokinins (CKs), gibberellins (GAs), ethylene, abscisic acid (ABA), and jasmonic acid (JA) (Bajguz and Piotrowska 2009;

Davies 2010; Piotrowska and Bajguz 2011; Ahanger et al. 2018; Šimura et al. 2018). The biological activity of plant hormones in plants is related to their concentration in cells and quantitative mutual ratios. The action of phytohormones mostly consists in regulating the metabolism of nucleic acids and proteins through their effect on replication, transcription, and translation processes (Weyers and Paterson 2001; Davies 2010). The level of endogenous phytohormone is the result of complex metabolic processes, such as biosynthesis, conjugation, degradation, and transport. These processes are subject to coordinated regulation, ensuring hormonal homeostasis, essential for the proper functioning of the plant. Phytohormones can interact with each other, increasing or decreasing the intensity of biological processes, or causing opposite effects on the same process. Hence, the following types of cooperation are distinguished: additive, synergistic, or antagonistic (Weyers and Paterson 2001; Gray 2004). Results obtained by Bajguz and Piotrowska-Niczyporuk

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(2013) confirmed that AXs with BRs cooperate synergistically, controlling the growth and metabolism of *Chlorella vulgaris*. In turn, 24-epibrassinolide (EBL) and indole-3-acetic acid (IAA) mixture has a synergistic effect on *Scenedesmus quadricauda* biomass production, the content of total carotenoids, chlorophyll *a*, and neutral lipid (triacylglyceride) (Kozlova et al. 2017; Kozlova et al. 2018). In the case of *Chlorella sorokiniana*, a combination of indole-3-butyric acid (IBA) and GA₃ also showed a synergistic effect on the growth; however, this did not occur when IAA was mixed with either GA₃ or kinetin (Ozioko et al. 2015).

Pollution of water with heavy metals, including lead (Pb), is a widespread problem of worldwide because they are non-biodegradable and tend to accumulate in living organisms (Long et al. 2014). Pb has been classified as a hazardous substance with high durability, which can elicit various biochemical, physiological, and morphological disorders in plants (Piotrowska-Niczyporuk et al. 2015; Bai et al. 2019). For example, high concentrations of heavy metal ions can breakdown protein synthesis, decline photosynthesis rates, as well as influence the activities of anti-oxidative enzymes, thus causing reduced plant resistances or tolerances to stress conditions (Song et al. 2012). Algae can accumulate Pb; hence, the biosorption has been used to control its content in aquatic ecosystems (Atici et al. 2010; Li et al. 2019). Nevertheless, Pb possesses a toxic effect on the growth and development of algae, and BRs can enhance the resistance of microalgae to heavy metals (Bajguz 2010; Talarek-Karwel et al. 2020). However, the study of Talarek-Karwel et al. (2020) demonstrated that 5-day exposure of *Acutodesmus obliquus* with 500 µM Pb is highly toxic (limited the growth), but is not deadly according to data from ECOTOX database, where 96 h LC₅₀ for the number of green algae species is within 0.5 to 10 mg L⁻¹ (United States Environmental Protection Agency 2020).

In plants growing under stress conditions, the concentrations of endogenous plant hormones may be lower, which is not enough to alleviate the negative effect of stress factors. Several reports confirmed that the adverse impact of heavy metals on plant growth and development was due to the decline in the endogenous level of phytohormones, which play an essential role in stress adaptation. Generally, the presence of heavy metal may lead to a decrease in endogenous levels of AXs, CKs, and GAs except for ABA, which endogenous content increases under stress conditions (Atanasova et al. 2004; Atici et al. 2005; Kim et al. 2014; Zelinová et al. 2015). For example, heavy metal stress declined the production and transport of CKs from roots via the xylem to shoot organs as well as less free forms of CKs, and more conjugates with less biological activity were detected in the shoots of *Juniperus communis* (Atanasova et al. 2004; Gangwar and Singh 2011).

Single-cell photosynthetic organisms, such as *A. obliquus*, are known for rapid biomass production and similarity with

vascular plants. Numerous studies confirmed the occurrence of AXs, CKs, GAs, BRs, and ABA and their significant influence on the growth and development of green algae from the *Chlorella* and *Acutodesmus* genus (Stirk et al. 2004; Stirk et al. 2009, 2013a, b, 2014a, b; Tarakhovskaya et al. 2007; Žižková et al. 2017; Bajguz 2019). Previous studies indicated that 1 µM EBL reduced the biosorption of Pb from the aquatic ecosystem by *A. obliquus* cultures and, therefore, improved algal growth as well as enhanced metal detoxification process (Talarek-Karwel et al. 2020). Thus, the exogenous application of BRs, especially EBL, characterized by high biological activity under heavy metal stress, may help restore phytohormone homeostasis and reduce unfavorable consequences of Pb on algal growth. Application of brassinolide (BL) indicated that the level of selected phytohormones (BL, IAA, zeatin, and ABA) was enhanced in *C. vulgaris* cultures exposed to heavy metals (cadmium, copper, and lead) action (Bajguz 2011). Hence, the aim of this study was to broaden previously published analyses/findings and extend the total amount of quantified phytohormones in *A. obliquus*. This alga, like other unicellular algae, is a suitable object of experiments on the direct action of exogenous growth substances, i.e., effect of EBL and/or Pb on the endogenous level of BRs, AXs, CKs, GA₃, and ABA. For that reason, the following hypotheses were tested: (1) Pb can decrease the endogenous level of the most phytohormones in *A. obliquus*, except for ABA, which is stress hormone; (2) EBL has a significant effect on the content of phytohormones in green alga; (3) EBL effectively alleviates the negative impact of Pb ions on the phytohormone homeostasis in algal cells. Obtained results should be important for better understanding of the role of BR in algal adaptation to heavy metal stress conditions.

Materials and methods

Test material and culture conditions

The SAG 276–6 strain of *A. obliquus*, initiated at 2×10^6 cells mL⁻¹, was cultivated in the Erlenmeyer flasks containing 100 mL of Bold Basal Medium (Andersen 2005; Andersen et al. 2005). Algal cultures were cultivated for 5 days in stable conditions of an air-conditioned room in a phytotron chamber at 25 ± 0.5 °C and under lights (photon flux 50 µmol photons m⁻² s⁻¹ at the surface of the tubes with a 16:8-h light/dark cycle and bubbling with air at 1 L min⁻¹).

According to Talarek-Karwel et al. (2018), the most stimulating effect of EBL (Sigma-Aldrich, USA) on algal growth was observed on the 5th day of cultivation at a concentration of 1 µM, whereas the highest toxicity of Pb in algal cultures was observed at a concentration of 500 µM. On the other hand, 0.01 µM Pb possessed the lowest toxic influence on *A. obliquus* growth and development. Hence, the level of

phytohormones was analyzed in algal cells treated with 0.01 μM and 500 μM Pb. Moreover, 1 μM EBL was used solely and in combination with 0.1 and 500 μM Pb to determine its impact on AXs, BRs, CKs, GA₃, and ABA homeostasis in *A. obliquus* cells on the 5th day of cultivation.

Phytohormones determination

To determine AXs, CKs, ABA, and GA₃, the algal precipitate was homogenized using a bead mill (50 Hz, 5 min; TissueLyser LT, Qiagen, Germany) in 1 mL of ice-cold 50% aqueous (v/v) acetonitrile (ACN). Then the samples were sonicated (3 min, 4 °C), shaken for 30 min at 4 °C (90 rpm, Laboratory shaker LC-350, Pol-Eko-Aparatura sp.J., Poland), and centrifuged (10 min, 9000×g, 4 °C). Before extraction, algal samples were collected by centrifugation (9000×g, 10 min).

The level of the following phytohormones, *trans*-zeatin (*tZ*), *trans*-zeatin-riboside (*tZR*), *trans*-zeatin-9-glucoside (*tZ9G*), *trans*-zeatin-7-glucoside (*tZ7G*), *trans*-zeatin-*O*-glucoside (*tZOG*), *trans*-zeatin-*O*-glucoside riboside (*tZROG*), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), dihydrozeatin-7-glucoside (DHZ7G), dihydrozeatin-9-glucoside (DHZ9G), *cis*-zeatin (*cZ*), *cis*-zeatin-riboside (*cZR*), *cis*-zeatin-*O*-glucoside (*cZOG*), *cis*-zeatin-*O*-glucoside riboside (*cZROG*), *N*⁶-isopentenyladenine (iP), *N*⁶-isopentenyladenosine (iPR), *N*⁶-isopentenyladenine-7-glucoside (iP7G), indole-3-acetic acid (IAA), ABA, GA₃, castasterone (CS), 24-epicastasterone (ECS), brassinolide (BL), EBL, 28-homobrassinolide (HBL), typhasterol (TY), was determined using appropriate labeled internal standards (1 pmol per standard) (OlChemIm s.r.o., Olomouc, Czech Republic).

The sample preparation with ion-exchange solid-phase extraction (SPE) was conducted according to Šimura et al. (2018) method using Oasis HLB RP polymer-based SPE cartridge (Waters, USA) which was activated and equilibrated with 100% methanol (MeOH), deionized H₂O, and 50% aqueous (v/v) ACN. After loading a supernatant, the flow-through fraction was collected in a glass tube. The cartridge was then rinsed with 30% (v/v) ACN, and this fraction was picked up to be dried by centrifugal evaporator (CentriVap IR Vacuum Centrifugal Concentrators, Labconco Corp., USA). After evaporated to dryness, samples dissolved in 30% ACN (v/v), transferred to chromatographic vials with inserts, and analyzed with LC-QTOF-MS method for determination of endogenous phytohormones' level. Agilent 1260 Infinity series HPLC system combined with 6540 UHD Accurate-Mass Q-TOF LC/MS mass spectrometer with Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) source was used to separate and identify of plant hormones in *A. obliquus*. HPLC system included an autosampler,

degasser, binary pump, and column oven with the Waters XSelect C₁₈ column (250 mm × 3.0 mm, 5 μm) kept at 50 °C. Two eluents were used for the separations of each sample (injection 10 μL): eluent A, 0.01% (v/v) formic acid (FA) in ACN and B, 0.01% (v/v) FA in H₂O at 0.5 mL min⁻¹ flow rate. The gradient was linear: 0–8 min, 5–30% A; 8–25 min, 80% A; 25–28 min, 100% A; and 28–30 min, 5% A.

The method of BRs purification and determination was employed, as described by Xin et al. (2013). BRs were extracted from frozen algal cells using in five portions of 1 mL MeOH in a bead mill (50 Hz, 5 min; TissueLyser LT, Qiagen, Germany). The obtained homogenates were centrifuged in the above conditions and collected between every homogenization. Next, the samples (each with a volume of 5 mL) were mixed (90 rpm, Laboratory shaker LC-350, Pol-Eko-Aparatura sp.J., Poland) for 12 h in the dark at 5 °C. The obtained supernatant was purified using Oasis MAX cartridge (6 mL, 500 mg; Waters, USA) which was activated and equilibrated with MeOH, water, 1 M KOH, 10% (v/v) MeOH, and 95% (v/v) MeOH in turn. The next, Oasis MCX cartridge (6 mL, 500 mg; Waters, Milford, USA) was activated and equilibrated with MeOH, water, 5% (v/v) FA and 10% (v/v) MeOH in turn. After that, algal extracts were let through a MAX cartridge and picked up to be dried by centrifugal evaporator (CentriVap IR Vacuum Centrifugal Concentrators). The next stage was the dissolution of the sample in 4 mL of 10% MeOH and passed it through a MCX cartridge. It was also necessary to sequentially wash the sample with 5% (v/v) FA in 5% (v/v) MeOH, 5% (v/v) MeOH, 5% (v/v) NH₄OH in 5% (v/v) MeOH and 5% (v/v) MeOH and finally BRs elution with 80% (v/v) MeOH. Samples thus prepared were transferred to chromatographic vials with inserts and analyzed using the LC-QTOF-MS system. HPLC system included the Waters XBridge C₁₈ column (250 mm × 4.6 mm, 1.7 μm) kept at 25 °C. Detection of BR-DMAPBA was possible by the use of a mass detector with Dual AJS ESI source. Mobile phase A was 0.1% (v/v) FA in water and B was 0.1% (v/v) FA in ACN at 0.2 mL min⁻¹ flow rate. Separation of the above hormones was done in ESI positive mode with following gradients: 0–2 min, 75% B; 2–14 min, 85% B; 14–40 min, 95% B; 40–42 min, 75% B; and 42,045 min, 75% B.

Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, USA). The MS parameters used for phytohormone detection and quantification were as follows: positive-ion mode, the nebulizer gas pressure (60 psi), drying gas pressure (50 psi), curtain gas pressure (30 psi), source voltage (3.5 kV), and source temperature at 350 °C. Nebulizer, drying, and collision gas was nitrogen. Agilent Mass-Hunter Workstation Software for LC/MS was used to instrument control and data processing.

Statistical analysis

The R software was used to perform statistical analyses (R Core Team 2019). Data grouped by phytohormone and treatment ($n = 4$) were subjected to the one-way ANOVA (“stats” package) followed by Tukey’s post hoc test (“laercio” package (da Silva 2010)). The Shapiro-Wilk test (“stats” package) and Levene’s test (“car” package (Fox and Weisberg 2019)) were used to verify ANOVA assumptions of normally distributed data with homogenous variances ($\alpha = 0.05$). Pearson’s correlations (“Hmisc” package (Harrell Jr. 2019)) were calculated to check the linear relationship between each phytohormone ($n = 24$), except DHZ7G that was not normally distributed (Table 1S). A resulting correlation matrix was visualized as a heatmap using the “corrplot” package (Wei and Simko 2017). Correlations were considered significant for $p < 0.05$.

Additionally, principal component analysis (PCA) was used to (1) transform multivariate data of phytohormones levels into a reduced form of new linearly uncorrelated factors (variables) and (2) explore underlying relations in the original dataset. The “FactoMineR” package was used for this purpose (Lê et al. 2008). All phytohormones’ values for each treatment ($n = 4$) were taken to the analysis. Data were scaled to unit variance (scale.unit = TRUE), and based on a percentage of explained variance criteria, the first two factors, explaining about 80.92% of the variance, were selected (Table 2S). The quality of phytohormones representation and the contribution to the principal compound was shown in Table 3S. A biplot was created using the “factoextra” package (Kassambara and Mundt 2019).

Results

The present study reports the occurrence of ABA, GA₃, AXs (IAA; phenylacetic acid, PAA), different types of BRs: 7-oxalactone type (BL, EBL, HBL), 6-oxo type (CS, ECS, TY, cathasterone (CT)), 6-deoxo type (6-deoxytyphasterol, dTY) as well as different types of CKs: free bases (*cZ*, *tZ*, DHZ, *iP*, *iPR*), *N*-glucosides (*tZ*7G, *tZ*9G, DHZ7G, DHZ9G, *iP*7G), *O*-glucosides (*cZ*OG, *cZ*ROG, *tZ*OG, *tZ*ROG, dihydrozeatin-*O*-glucoside (DHZOG)), and ribosides (*cZ*R, *tZ*R, DHZR) in *A. obliquus* (Table 1). In terms of quantity (overall mean), AXs dominated in green alga (25.93 pmol PAA g⁻¹ fresh weight), slightly lower content was detected for CKs (7.96 pmol *cZ* g⁻¹ fresh weight), ABA (5 pmol g⁻¹ fresh weight), BRs (3.25 pmol CT g⁻¹ fresh weight), and GA₃ (1.76 pmol g⁻¹ fresh weight).

The content of detected BRs in *A. obliquus* was arranged in the following order: CT > BL > CS > EBL > TY > dTY > ECS > HBL (Table 1). The highest inhibitory effect of Pb on the BRs level was observed at a concentration of 500 μM. For example, heavy metal stress declined BL level by 38% and

HBL by 90%. The maximum increase in BRs level was 93% for CT, 84% for EBL, 78% for ECS, 73% for BL, 70% for HBL, 70% for dTY, 61% for TY, and 32% for CS after application of 1 μM EBL in relation to control. *A. obliquus* cells treated with both Pb and 1 μM EBL were characterized by the increase in BRs content by 56% and 61% (in the case of ECS and EBL, respectively) in relation to the control. EBL also alleviated the toxic effect of Pb on the content of other BRs, which levels were close to the values observed in control cultures.

Compared with unstressed plants, a significant (118%) increase in the content of ABA was observed in the presence of 500 μM Pb (Table 1). On the other hand, 1 μM EBL caused the decline in the content of this phytohormone by 35% in comparison with the control. The co-application of Pb with EBL did not possess a statistically significant influence on the ABA level in *A. obliquus* cells.

The reduction in the content of GA₃ was detected in *A. obliquus* cultures exposed to Pb (Table 1). The highest (45%) decrease in the GA₃ level was reported after the application of 500 μM Pb in relation to the control group. Among the tested conditions, the application of 1 μM EBL alone had the most stimulating effect, inducing an 80% rise in GA₃ concentration in algal cells in comparison with the control. EBL alleviated the inhibitory effect of Pb, causing 41% increase in GA₃ content after 0.01 μM Pb and 1 μM EBL application and maintaining GA₃ at the control level after treatment with 500 μM Pb and 1 μM EBL.

A higher PAA level than IAA in *A. obliquus* was noted (Table 1). Under 500 μM Pb exposure, the significant decline in the AXs content (by 44% for IAA and 46% for PAA) in relation to the control was observed in algal cultures. EBL at a concentration of 1 μM enhanced the level of PAA by 69% and IAA by 66% in comparison with the control. The application of EBL reversed the inhibitory effect of Pb on AXs level, resulting in 34–52% increase in the content of analyzed AXs (1 μM EBL + 0.01 μM Pb) or maintaining their concentrations at the control level (1 μM EBL + 500 μM Pb).

Free bases (43–66%) and *cZ*-type (49–60%) of CKs possessed the highest share among all detected types of CKs in *A. obliquus* cells (Fig. 1). In turn, the smallest part was recorded in the case of *N*-glucosides (3–8%) and *tZ*-type (8–16%) of CKs. The level of isoprenoid type of CKs in green alga was in the following order: *cZ*-type > *iP*-type > DHZ-type > *tZ*-type. Among the free bases, ribosides, and conjugates, the hierarchy of CKs level was free bases > *O*-glucosides > ribosides > *N*-glucosides. Among CKs, the levels of free bases and ribosides were the most reduced in cells subjected to Pb stress at a concentration of 500 μM. In algal cells treated with 500 μM Pb, remarkable decreases in CKs by 51% for *cZ*, 39% for *iP*, 49% for *iPR*, 59% for DHZ, 71% for *tZ*, 48% for *cZ*R, 50% DHZR, and 83% for *tZ*R were reported in relation to the control cultures. The application of 1 μM EBL to algal

Table 1 The concentrations of phytohormones (pmol g⁻¹ fresh weight) in *Acutodesmus obliquus* treated with Pb and/or EBL

	Control	0.01 μ M Pb	500 μ M Pb	1 μ M EBL	0.01 μ M Pb + 1 μ M EBL	500 μ M Pb + 1 μ M EBL	Overall mean
ABA	3.97 $\pm$ 1.28 ^{bc}	5.29 $\pm$ 0.71 ^b	8.67 $\pm$ 0.69 ^a	2.57 $\pm$ 0.58 ^c	4.23 $\pm$ 1.01 ^{bc}	5.27 $\pm$ 1.00 ^b	5.00
GA ₃	1.57 $\pm$ 0.24 ^{cd}	1.24 $\pm$ 0.06 ^{de}	0.87 $\pm$ 0.09 ^e	2.81 $\pm$ 0.32 ^a	2.22 $\pm$ 0.28 ^b	1.85 $\pm$ 0.14 ^{bc}	1.76
IAA	17.74 $\pm$ 1.15 ^b	12.96 $\pm$ 1.10 ^c	9.89 $\pm$ 1.17 ^c	29.36 $\pm$ 3.07 ^a	26.93 $\pm$ 1.05 ^a	21.36 $\pm$ 1.72 ^b	19.71
PAA	24.75 $\pm$ 3.05 ^c	17.29 $\pm$ 0.96 ^d	13.15 $\pm$ 1.54 ^d	41.73 $\pm$ 3.00 ^a	33.18 $\pm$ 3.38 ^b	25.49 $\pm$ 2.35 ^c	25.93
CS	1.94 $\pm$ 0.36 ^b	1.16 $\pm$ 0.18 ^c	1.08 $\pm$ 0.15 ^c	2.56 $\pm$ 0.22 ^a	2.12 $\pm$ 0.14 ^{ab}	1.87 $\pm$ 0.19 ^b	1.79
ECS	0.18 $\pm$ 0.04 ^{cd}	0.10 $\pm$ 0.02 ^{de}	0.07 $\pm$ 0.03 ^e	0.32 $\pm$ 0.05 ^a	0.28 $\pm$ 0.04 ^{ab}	0.20 $\pm$ 0.06 ^{bc}	0.19
BL	2.77 $\pm$ 0.17 ^{bc}	1.98 $\pm$ 0.41 ^{cd}	1.72 $\pm$ 0.43 ^d	4.80 $\pm$ 0.19 ^a	3.47 $\pm$ 0.55 ^b	2.88 $\pm$ 0.20 ^b	2.94
EBL	0.49 $\pm$ 0.14 ^c	0.26 $\pm$ 0.06 ^{cd}	0.16 $\pm$ 0.05 ^d	0.90 $\pm$ 0.14 ^a	0.79 $\pm$ 0.19 ^{ab}	0.51 $\pm$ 0.12 ^{bc}	0.52
HBL	0.10 $\pm$ 0.03 ^b	0.03 $\pm$ 0.04 ^{cd}	0.01 $\pm$ 0.01 ^d	0.17 $\pm$ 0.03 ^a	0.15 $\pm$ 0.03 ^{ab}	0.09 $\pm$ 0.03 ^{bc}	0.09
CT	3.00 $\pm$ 0.41 ^{bc}	2.82 $\pm$ 0.28 ^c	1.14 $\pm$ 0.26 ^d	5.79 $\pm$ 0.37 ^a	3.70 $\pm$ 0.32 ^b	3.03 $\pm$ 0.23 ^{bc}	3.25
TY	0.23 $\pm$ 0.05 ^{bc}	0.16 $\pm$ 0.02 ^{cd}	0.11 $\pm$ 0.04 ^d	0.37 $\pm$ 0.05 ^a	0.30 $\pm$ 0.04 ^{ab}	0.27 $\pm$ 0.04 ^b	0.24
dTY	0.23 $\pm$ 0.05 ^{bc}	0.18 $\pm$ 0.03 ^{cd}	0.10 $\pm$ 0.03 ^d	0.39 $\pm$ 0.06 ^a	0.31 $\pm$ 0.04 ^{ab}	0.27 $\pm$ 0.05 ^{bc}	0.25
cZ	7.82 $\pm$ 1.00 ^{bc}	4.99 $\pm$ 1.13 ^{cd}	3.82 $\pm$ 0.59 ^d	12.45 $\pm$ 2.02 ^a	10.24 $\pm$ 2.19 ^{ab}	8.45 $\pm$ 1.20 ^b	7.96
cZR	2.69 $\pm$ 0.28 ^{bc}	2.08 $\pm$ 0.30 ^c	1.40 $\pm$ 0.23 ^d	3.61 $\pm$ 0.27 ^a	3.01 $\pm$ 0.12 ^{ab}	2.08 $\pm$ 0.38 ^c	2.48
cZOG	0.62 $\pm$ 0.20 ^c	1.03 $\pm$ 0.13 ^{ab}	1.28 $\pm$ 0.20 ^a	0.85 $\pm$ 0.07 ^{bc}	0.68 $\pm$ 0.18 ^c	0.87 $\pm$ 0.09 ^{bc}	0.89
cZROG	0.42 $\pm$ 0.14 ^b	0.59 $\pm$ 0.12 ^{ab}	0.82 $\pm$ 0.14 ^a	0.72 $\pm$ 0.12 ^{ab}	0.48 $\pm$ 0.14 ^b	0.61 $\pm$ 0.15 ^{ab}	0.61
tZ	0.42 $\pm$ 0.12 ^{bc}	0.21 $\pm$ 0.05 ^{de}	0.12 $\pm$ 0.03 ^e	0.72 $\pm$ 0.06 ^a	0.54 $\pm$ 0.05 ^b	0.34 $\pm$ 0.05 ^{cd}	0.39
tZR	0.12 $\pm$ 0.03 ^b	0.06 $\pm$ 0.02 ^{bc}	0.02 $\pm$ 0.02 ^c	0.21 $\pm$ 0.03 ^a	0.22 $\pm$ 0.05 ^a	0.12 $\pm$ 0.03 ^b	0.12
tZOG	0.81 $\pm$ 0.08 ^c	1.30 $\pm$ 0.19 ^b	1.65 $\pm$ 0.15 ^a	1.08 $\pm$ 0.15 ^{bc}	0.85 $\pm$ 0.08 ^c	1.27 $\pm$ 0.11 ^b	1.16
tZROG	0.07 $\pm$ 0.03 ^b	0.17 $\pm$ 0.04 ^a	0.19 $\pm$ 0.03 ^a	0.10 $\pm$ 0.03 ^b	0.09 $\pm$ 0.03 ^b	0.13 $\pm$ 0.03 ^{ab}	0.12
tZ7G	0.03 $\pm$ 0.03 ^b	0.09 $\pm$ 0.02 ^{ab}	0.14 $\pm$ 0.04 ^a	0.07 $\pm$ 0.03 ^b	0.08 $\pm$ 0.02 ^{ab}	0.08 $\pm$ 0.03 ^{ab}	0.08
tZ9G	0.14 $\pm$ 0.02 ^c	0.24 $\pm$ 0.03 ^{ab}	0.26 $\pm$ 0.06 ^a	0.21 $\pm$ 0.04 ^{abc}	0.19 $\pm$ 0.03 ^{abc}	0.16 $\pm$ 0.04 ^{bc}	0.20
DHZ	0.75 $\pm$ 0.17 ^{bc}	0.52 $\pm$ 0.07 ^{cd}	0.31 $\pm$ 0.04 ^d	1.09 $\pm$ 0.17 ^a	0.87 $\pm$ 0.07 ^{ab}	0.90 $\pm$ 0.11 ^{ab}	0.74
DHZR	1.15 $\pm$ 0.15 ^{bc}	0.79 $\pm$ 0.12 ^{de}	0.57 $\pm$ 0.08 ^e	1.55 $\pm$ 0.16 ^a	1.40 $\pm$ 0.14 ^{ab}	1.00 $\pm$ 0.10 ^{cd}	1.08
DHZOG	0.28 $\pm$ 0.07 ^b	0.39 $\pm$ 0.10 ^{ab}	0.55 $\pm$ 0.08 ^a	0.35 $\pm$ 0.05 ^b	0.33 $\pm$ 0.05 ^b	0.44 $\pm$ 0.09 ^{ab}	0.39
DHZ7G	0.08 $\pm$ 0.01 ^b	1.02 $\pm$ 0.10 ^a	1.15 $\pm$ 0.06 ^a	0.35 $\pm$ 0.49 ^b	0.09 $\pm$ 0.01 ^b	1.10 $\pm$ 0.03 ^a	0.63
DHZ9G	0.09 $\pm$ 0.02 ^c	0.18 $\pm$ 0.02 ^{ab}	0.23 $\pm$ 0.04 ^a	0.17 $\pm$ 0.05 ^{abc}	0.13 $\pm$ 0.03 ^{bc}	0.13 $\pm$ 0.03 ^{bc}	0.15
iP	1.76 $\pm$ 0.16 ^c	1.30 $\pm$ 0.26 ^{cd}	1.07 $\pm$ 0.19 ^d	3.33 $\pm$ 0.22 ^a	2.31 $\pm$ 0.26 ^b	1.75 $\pm$ 0.22 ^c	1.92
iPR	2.14 $\pm$ 0.14 ^{bc}	1.63 $\pm$ 0.20 ^d	1.10 $\pm$ 0.12 ^e	3.65 $\pm$ 0.16 ^a	2.43 $\pm$ 0.22 ^b	1.89 $\pm$ 0.16 ^{cd}	2.14
iP7G	0.17 $\pm$ 0.02 ^b	0.24 $\pm$ 0.05 ^{ab}	0.28 $\pm$ 0.04 ^a	0.22 $\pm$ 0.03 ^{ab}	0.16 $\pm$ 0.03 ^b	0.17 $\pm$ 0.04 ^b	0.21

Data, grouped by treatment for each phytohormone, represent the mean ($n = 4$) $\pm$ standard deviation. The same letters indicate statistically nonsignificant differences according to Tukey's post hoc test ($p \geq 0.05$)

cultures induced an increase in the concentration of iP, iPR, tZ, tZR, cZ, cZR, DHZ, and DHZR (by 89%, 71%, 71%, 75%, 59%, 34%, 45%, and 35%, respectively) in relation to the control group. The combined treatment of algal cells with EBL + Pb enhanced the content of CKs. On the other hand, Pb at the concentration of 500 μ M caused the most significant increase in *N*- and *O*-glucosides conjugates of CKs compared with non-Pb treated algal cells. The level of *N*- and *O*-glucosides decreased after treatment with EBL alone and after the co-application with Pb.

Correlation analysis of phytohormones in *A. obliquus* revealed that ABA is the only phytohormone strongly or very strongly, negatively correlated (according to Evans (1996)) with all studied AXs and BRs as well as GA₃ and some of CKs, e.g., cZ, cZR, and tZ (Fig. 2). Moreover, almost all

studied AXs, and BRs show a very strong, positive correlations between each other. cZ, cZR, tZ, tZR, DHZ, DHZR, iP, and iPR have very strong, positive correlations with GA₃, AXs, and BRs, as well as among themselves. cZOG, tZOG, tZROG, tZ7G, and DHZOG showed mostly moderate and negative correlations with GA₃, AXs, BRs, cZ, cZR, tZ, tZR, and between each other. Furthermore, very strong and positive correlations were found: tZ vs. tZR, DHZR, iP, and iPR; tZR vs. DHZR and iP; DHZ vs. DHZR and iP; DHZR vs. iP and iPR; and iP vs. iPR.

Positive scores for the first principal component (Dim1) indicate higher values of GA₃, IAA, PAA, CS, ECS, BL, EBL, HBL, CT, TY, dTY, cZ, cZR, tZ, tZR, DHZ, DHZR, iP, and iPR, while negative show higher values of the rest of studied phytohormones, compared with the mean values for

Fig. 1 The percentage share of different types of cytokinins in *Acutodesmus obliquus* treated with lead and 24-epibrassinolide (EBL). **a** Free bases (cZ, tZ, DHZ, iP, iPR) and conjugates, N-glucosides (tZ7G, tZ9G, DHZ7G, DHZ9G, iP7G), O-glucosides (cZOG, cZROG, tZOG, tZROG),

tZROG, DHZOG), and ribosides (cZR, tZR, DHZR); **b** Isoprenoid type, cZ-type (cZ, cZOG, cZROG, cZR), tZ-type (tZ, tZR, tZ7G, tZ9G, tZOG, tZROG), and DHZ-type (DHZ, DHZR, DHZOG, DHZ7G, DHZ9G), iP-type (iP, iPR, iP7G)

all studied algal treatments (Fig. 3). The phytohormones show a very good quality of representation on the created model. PCA did not cluster any algal treatments. Based on treatments, increase of the values of GA₃, IAA, PAA, CS, ECS, BL, EBL, HBL, CT, TY, dTY, cZ, cZR, tZ, tZR, DHZ, DHZR, iP, and iPR is in the following order: 1 μ M EBL > 0.01 μ M Pb + 1 μ M EBL > 500 μ M Pb + 1 μ M EBL > control > 0.01 μ M Pb > 500 μ M Pb. On the other hand, increase of cZOG, cZROG, tZOG, tZROG, tZ7G, tZ9G, DHZOG, DHZ7G, DHZ9G, and iP7G values is in the treatment with 500 μ M Pb > 0.01 μ M Pb > 500 μ M Pb + 1 μ M EBL > 1 μ M EBL > 0.01 μ M Pb + 1 μ M EBL > control; for ABA, 500 μ M Pb > 0.01 μ M Pb > 500 μ M Pb + 1 μ M EBL > 0.01 μ M Pb + 1 μ M EBL > control > 1 μ M EBL; for cZROG, 500 μ M Pb > 1 μ M EBL > 500 μ M Pb + 1 μ M EBL > 0.01 μ M Pb > 0.01 μ M Pb + 1 μ M EBL > control. Thus, Pb alone limits the content of GA₃, AXs, BRs, free bases, and ribosides conjugates of CKs and significantly increases the level of other phytohormones (ABA and N-glucosides, O-glucosides of CKs). On the other hand, EBL alone has the opposite effect. Furthermore, the application of EBL reduces limitations in BRs, GA₃, AXs, and selected CKs level in the cultures subjected to Pb stress.

Discussion

Phytohormones and their homeostasis play key roles in many aspects of plant growth and development. Moreover, plant hormones are engaged in biotic and abiotic stress responses. The obtained results showed that after Pb treatment, the contents of BRs, GA₃, AXs, free bases of CKs, and ribosides of CKs were significantly decreased in green alga *A. obliquus* (Table 1). Pb is a phytotoxic heavy metal, and its inhibitory

effects on plant growth and development may be connected with its effect on the level of phytohormones involved in cell proliferation and enlargement. Previous studies demonstrated that Pb inhibited *A. obliquus* growth and primary metabolite accumulation and generated oxidative stress (Piotrowska-Niczyporuk et al. 2015). In turn, it was found that Cu excess causes changes in the distribution of AXs and CKs in *Arabidopsis thaliana* roots (Lequeux et al. 2010). It has also been observed that cadmium (Cd) stress caused a decline AXs level in wood and thereby shunted the metabolism to increased formation of lignin in poplar (Elobeid et al. 2012). These and other studies present that except for some biological processes, such as changes in cell division and elongation, growth, nutrient uptake, and reactive oxygen species level, alterations in hormonal homeostasis may be involved in response to heavy metal stresses.

The presence of the most widely distributed BRs, which is CS followed by BL, TY, 6-deoxoCS, teasterone (TE), and 28-norCS, was confirmed in angiosperms, gymnosperms, algae, bryophyte and pteridophyte (Stirk et al. 2013a; Kanwar et al. 2017; Yokota et al. 2017; Bajguz 2019). In the case of algae, several reports confirm the endogenous occurrences of BRs. BRs are intermediates in the early and late C6-oxidation pathways; therefore, BRs such as BL and CS were identified in 24 microalgae strains (Stirk et al. 2013a). BRs such as TE, TY, 6-deoxoTE, 6-deoxoTY, 6-deoxoCS, CS, and BL were detected in *Chlorella vulgaris* cells (Bajguz 2009b). Moreover, green alga *Hydrodictyon reticulatum* contains endogenous ECS and 28-homoCS (Yokota et al. 1987), whereas BL and CS were isolated from kelp *Ecklonia maxima* (Stirk et al. 2014b). Present studies demonstrate that exogenous EBL was characterized by a significant effect on the biosynthesis of BRs in *A. obliquus*, increasing the level of different types of BRs

Fig. 2 Correlation heatmap for phytohormones ($n = 24$) in *Acutodesmus obliquus*. The colored and labeled scale codes for the value of Pearson's correlation coefficient r . Positive correlations are green, while negative correlations are red. Gray crosses show nonsignificant correlations ($p \geq 0.05$)

(Table 1). Furthermore, previous research also points out that exogenous EBL is an effective stimulator of *A. obliquus* growth as it causes an increase in the number of cells and the contents of selected metabolites. EBL protects against the effects of oxidative stress and activates antioxidant systems in the green algae (Talarek-Karwel et al. 2018). Thus, exogenous EBL has a positive impact on the BRs level in green alga and additionally protects plants against the harmful effects of heavy metal. The reduction of BRs content in green

alga treated with Pb was prevented by EBL (Table 1). The co-application of EBL with Pb showed a significant increase in the BRs concentration when compared with cultures treated with Pb alone. The protective role of EBL against Pb stress in *A. obliquus* cultures may be explained by inhibition of metal bioaccumulation which allows cells to keep precise homeostatic regulation of intracellular heavy metal level (Talarek-Karwel et al. 2020) and by the increase in the endogenous level of BRs which are involved in algal resistance against

BRs control numerous biological processes by interplay with many phytohormones, i.e., GAs, AXs, CKs, ABA, and salicylic acid (Hao et al. 2013; Arif et al. 2020; Sharma et al. 2020). Several interesting networking mechanisms, such as protein-protein interaction and transcription, are responsible for the regulation of coordination between BRs and other plant hormones (Ahanger et al. 2018). Many studies confirm the fundamental interactions of BRs with phytohormones, uniquely synergistic, and antagonistic (Bai et al. 2012; Bajguz and Piotrowska-Niczyporuk 2013, 2014; Bhardwaj et al. 2014). Firm evidence exists that BRs are also responsible for heavy metal stress tolerance mechanisms in plants (Talarek-Karwel et al. 2018). Exogenous application of BRs may result in interplay with other phytohormones and co-regulation of physio-biochemical processes in plants to counteract the stress-induced changes and to enhance the effectiveness of hormones in regulating stress tolerance (Ahanger et al. 2018).

BRs and GAs regulate photomorphogenesis, cell elongation, seed germination, and flowering. A connection between BRs and GA is associated with the DELLA-BZR-PIF module. This module can regulate a broad spectrum of light-response components. Moreover, repressors of GAs responses, DELLA proteins may be engaged in stress avoidance (Bai et al. 2012). Stirk et al. (2013a) confirmed the occurrence of endogenous GAs in 24 axenic microalgae strains from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Charophyceae. Several researchers also showed the positive impact of GAs on green algae. For example, exogenous GAs improved the growth rate of *Chlamydomonas reinhardtii* and stimulated astaxanthin biosynthesis in *Haematococcus pluvialis* (Lu et al. 2010; Park et al. 2013; Lu and Xu 2015). The obtained results suggest that the level of GA₃ changed in *A. obliquus* cells after the treatment with Pb and/or EBL. Heavy metal excess caused the weakness ability of *A. obliquus* cells to the biosynthesis of GAs. Similarly, higher concentrations of heavy metals (Pb, Zn) decreased the GA₃ level in the chickpea germinating seed (Atici et al. 2005). Under other stress conditions like salinity stress, a decline in the endogenous level of GA₃ in soybean has been observed (Hamayun et al. 2010). The present study confirms that the addition of exogenous EBL might be useful to induce the biosynthesis of GAs (Table 1). The co-application of EBL and Pb led to an increase in the content of GA₃ in relation to cultures treated with heavy metal alone.

BRs and AXs may act synergistically to enhance plant growth responses and transcriptional regulation. BRs can increase AXs-induced growth responses, hypocotyl elongation, root development, shoot gravitropism, and laminar inclination (Vandenbussche et al. 2013). Furthermore, the presence of a lot of the common genes that may be induced by both phytohormones was observed in *A. thaliana* (Hao et al. 2013).

The occurrence of natural AXs, including IAA, PAA, and indole-3-butyric acid (IBA), were detected in Chlorophyceae (Stirk et al. 2004, 2009, 2013b; Žižková et al. 2017). The concentration of active free IAA is controlled by the rate of biosynthesis, conversion into storage conjugates, and degradation. IAA is synthesized via the indole-3-acetamide pathway in many microalgal strains (Stirk et al. 2013b). Present studies confirmed that Pb significantly reduced the concentration of AXs in *A. obliquus* cells (Table 1). Generally, heavy metal stress leads to a decrease in endogenous levels of AXs. Similar results also showed that arsenic (As) stress declined the levels of IAA and IBA in *Brassica juncea* (Srivastava et al. 2012). In the case of barley, short-term Cd action disturbed IAA homeostasis in root tips (Zelinová et al. 2015). The present study revealed the significant effect of 1 μM EBL on the rise in the level of PAA and IAA in *A. obliquus* cultures subjected to Pb stress. However, the most stimulating effect of BR on the concentration of

AXs was detected after the application of EBL alone (Table 1).

Recent investigations have proved CKs crosstalk with other hormones either by direct cooperation for inducing a typical growth response or imparting indirect, interactive impact reflecting the role of other plant hormones (Ahanger et al. 2018). Bajguz and Piotrowska-Niczyporuk (2014) indicated that CKs might stimulate BRs level and activity in green alga *C. vulgaris*. Isoprenoid CKs are divided into four groups, *tZ*, *cZ*, *iP*, and *DHZ*, with aromatic CKs and topolins (Stirk et al. 2009). Conjugates of these free bases include ribotides (riboside-5'-phosphates), ribosides, *O*-glycosides, *N*-glycoside, and amino acid conjugates. CKs free bases are usually the most biologically active in bioassays. In the case of *O*-glucosides, their level in algae changes from moderate to low depending on the age of the culture (Stirk et al. 2009; Stirk et al. 2013b). The *O*-glucosides are used as slow-release storage forms that refill the active aglycons to provide a constant supply of free CKs over an extended period. Furthermore, *O*-glucosides have a significant role in controlling CKs activity in tissues with a high level of CK oxidase because they are refractory to degradation by CK oxidase (Ördög et al. 2004). The occurrence of basic CKs in green microalgae (*Protococcus*, *Chlorella*, and *Scenedesmus*), out of which *cZ*-type were prevalent, has also been reported (Stirk et al. 2004, 2013b, 2014a).

Among all identified CKs in *A. obliquus* cells, *cZ*-type of CKs occurred at the highest level. Moderate concentrations of *iP*-type of CKs, low levels of *tZ*-type, and *DHZ*-type of CKs were confirmed. *O*- and *N*-glucosides of CKs were detected in very low concentrations in algal cells (Fig. 1). Interestingly, obtained results showed that Pb increased the level of *N*- and *O*-glucosides of CKs in *A. obliquus* culture (Table 1). In the presence of a high level of heavy metals (Pb, Cu, Zn, and Al) and also heat stress, the ratio of CKs conjugation process with glucose and xylose increases (Bajguz and Piotrowska 2009). *Acutodesmus obliquus* cells subjected to heavy metal stress (500 μM Pb) can accumulate higher levels of *N*-glucosides (especially *DHZ7G*) in comparison with free bases of CKs. A significant increase in the concentrations of conjugated forms of CKs under heavy metal stress indicated that CKs could also act as stress molecules in green algae. According to Stirk et al. (2004) high temperature led to elevated CKs contents in the seaweeds *E. maxima* and *Macrocystis pyrifera*. In turn, stress-induced CKs synthesis in the rice promoted sink intensive through a CKs-dependent coordinated regulation of nitrogen and carbon metabolism that increased tolerance of the rice to water deficit (Reguera et al. 2013). Obtained results also indicated that exogenous EBL enhanced the level of the free bases and ribosides of CKs (Table 1). EBL, as the regulator of the cellular response to heavy

metal stress, also reversed the negative effect of Pb on the above CKs. It can be speculated that BRs may increase *A. obliquus* tolerance to heavy metal stress by stimulating the accumulation of endogenous CKs. Other studies also clearly indicated the ameliorative effect of CKs on the resistance of green alga *C. vulgaris* and improvement of growth in the presence of heavy metals (Cd, Pb, Cu) (Piotrowska-Niczyporuk et al. 2012).

Both BRs and ABA are stress hormones that control numerous developmental processes, including seed development, dormancy, germination, vegetative growth, and environmental stress responses. ABA concentration in plants rises in response to abiotic stresses, such as heavy metals, drought, freezing, or heat, causing characteristic biochemical responses. Different factors, like the rate of synthesis, conjugation, tissue susceptibility, oxidative degradation, as well as long-distance transport, determine the ABA level at the site of action (Piotrowska and Bajguz 2011). Previous investigations reported that ABA is synthesized in green algae such as *C. vulgaris*, *Ulva fasciata*, *C. minutissima*, and brown alga *Dictyota humifusa* in relatively low concentrations. Furthermore, ABA biosynthesis appears to be similar in algae and vascular plants, based on the presence of homologous enzymes (Bajguz 2009a; Stirk et al. 2014a, b). The present research showed that under Pb-stress, green alga *A. obliquus* synthesized much more ABA than unstressed plants (Table 1). The increase in ABA level was also observed in green algae *Dunaliella* sp. and *C. reinhardtii* under salt stress and in *H. pluvialis* exposed to dehydration as well as in *C. reinhardtii* under osmotic and oxidative stress (Lu and Xu 2015). Similar results were also obtained in the case of higher plants. For example, the application of Cd leads to an increase in endogenous ABA concentration in potato (*Solanum lycopersicum*) tubers (Stroiński et al. 2010) and in rice (*Oryza sativa*) (Kim et al. 2014). The increase in ABA concentration was also obtained in germinating chickpea (*Cicer arietinum*) seeds under Pb toxicity conditions (Atici et al. 2005). In turn, the inhibition of ABA synthesis and the decline in the content of this phytohormone were observed in *A. obliquus* cultures after the application of EBL (Table 1). BRs possess the antagonistic effect on ABA synthesis and plant responses. The antagonistic effect of ABA on BRs signaling is based on the phosphorylation state of BR-INSSENSITIVE1-EMS-SUPPRESSOR1 transcription factor of the BR signaling pathway, plants-specific transcription factor, which can regulate the BRs synthesis. The sensitivity of BR to ABA during seed germination suggests that BR can regulate the same components (Bhardwaj et al. 2014). On the other hand, the study by Bajguz (2009a) on *C. vulgaris* cultures subjected to short-term (3 h) heat stress (30–40 °C) demonstrated that BL enhanced the content of ABA with increasing the temperature leading to higher algal tolerance to abiotic stress.

Conclusions

The study presents the occurrence of 30 phytohormones for the first time in *A. obliquus*, which content based on PCA and ANOVA was arranged in the following order: AXs > CKs > ABA > BRs > GA₃. The changes in the endogenous hormone level in *A. obliquus* after the application of exogenous EBL and/or Pb were reported. EBL alone increased the content of BRs, GA₃, AXs, and CKs (free bases and ribosides). Pb inhibited the levels of BRs, GA₃, AXs, free bases of CKs, and CK ribosides, whereas increased ABA, and *N*- and *O*-glucosides of CKs. ABA was reported to be the only phytohormone strongly or very strongly, negatively correlated with all BRs and AXs, as well as GA₃ and some of the CKs, further confirming the known BRs antagonistic effect on ABA synthesis. Furthermore, exogenous EBL effectively alleviated the negative impact of Pb on the content of phytohormones leading to higher algal resistance to stress conditions.

Authors' contributions Concept of the study, AB; collection and assembly of data, MT-K; drafting of the article, MT-K; interpretation of the data, MT-K, AP-N; statistical analysis, AB; critical revision and valuable intellectual content, AB; final approval of the article, all authors.

Compliance with ethical standards

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