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Effect of Cadmium on the Level of Isoprenoid-Derived Phytohormones in Duckweed *Wolffia arrhiza*

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Abstract

Wolffia arrhiza (L.) Horkel ex Wimm. is an aquatic plant belonging to the Lemnaceae family. It does not have leaves, stems, and roots, flowers rarely occur, while body size can reach 1 mm of width and 1.3 mm of length. The present study demonstrates the endogenous level of isoprenoid-derived phytohormones and their changes under the influence of different cadmium (Cd) concentrations (0.1, 1, 10, and 100 µM). A liquid chromatography quadrupole-time-of-flight mass spectrometry analysis indicated the presence of abscisic acid, eight brassinosteroids (6-deoxocastasterone, 6-deoxotyphasterol, cathasterone, typhasterol, castasterone, 24-epicastasterone, brassinolide, and 28-homobrassinolide), seven free bases of cytokinins [*trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), dihydrozeatin (DHZ), N^6-isopentenyladenine, N^6-isopentenyladenosine, *ortho*-topolin, and *meta*-topolin], eight conjugates of cytokinins (*t*Z riboside, *t*Z-9-glucoside, *t*Z-7-glucoside, *t*Z-*O*-glucoside riboside, *c*Z-9-glucoside, DHZ riboside, DHZ-*O*-glucoside, and N^6-isopentenyladenosine-7-glucoside) and gibberellic acid (GA_3) in this duckweed. The level of phytohormones in plants treated with Cd has changed, e.g., the ABA level increased while GA_3 decreased. Whereas the amount of BRs and CKs was different in Cd dose-dependent manner. Besides, it is worth noting that the distribution of 25 various phytohormones in the *Wolffia arrhiza* is reported for the first time.

Keywords Abscisic acid · Brassinosteroids · Cadmium stress · Cytokinins · Gibberellic acid · Occurrence

Abbreviations

EBL	24-Epibrassinolide
ECS	24-Epicastasterone
HBL	28-Homobrassinolide
6dCS	6-Deoxocastasterone
6dTY	6-Deoxytyphasterol
ABA	Abscisic acid
BL	Brassinolide
BR	Brassinosteroid
CS	Castasterone
CT	Cathasterone
CK	Cytokinin
*c*Z	*cis*-Zeatin
DHZ	Dihydrozeatin
DHZOG	Dihydrozeatin-*O*-glucoside
DHZR	Dihydrozeatin riboside
GA	Gibberellin
GA_3	Gibberellin A_3
iP	N^6-Isopentenyladenine
iPR	N^6-Isopentenyladenosine
iPR7G	N^6-Isopentenyladenosine-7-glucoside
*t*Z	*trans*-Zeatin
*t*Z7G	*trans*-Zeatin-7-glucoside
*t*Z9G	*trans*-Zeatin-9-glucoside
*t*ZROG	*trans*-Zeatin-*O*-glucoside riboside
*t*ZR	*trans*-Zeatin-riboside
TY	Typhasterol

Introduction

The Lemnaceae (duckweed) comprises an aquatic monocotyledon family, including only 37 species arranging within five genera and in majority widely distributing in the world. They are the smallest angiosperms, some of which may attain a width of only 0.3 mm at maturity (Les et al. 2002; Sree et al. 2016). Among Lemnaceae and other higher

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plants, *Wolffia arrhiza* (L.) Horkel ex Wimm. has significantly reduced organs, it does not have a stem, leaves, and root system. Body size can reach 1 mm of width and 1.3 mm of length, while flowering occurs extremely rarely (Schmitz and Kelm 2017; Khvatkov et al. 2018). Despite simplified body structure, these plants play crucial roles in the protection of the aquatic environment. In organic-rich water, they change the feeding from photoautotrophic into either mixotrophic or heterotrophic. Furthermore, duckweeds can bioaccumulate heavy metals and xenobiotics from polluted waters (Les et al. 1997; Vermaat and Hanif 1998; Samardakiewicz and Woźny 2000; Piotrowska et al. 2010; Soda et al. 2013). Duckweeds are applicable in aquaculture as food for waterfowl and fish. The simplified morphology of the plant enables it to be a good model for laboratory studies (Skillicorn et al. 1993; Piotrowska and Bajguz 2012).

Abscisic acid (ABA), brassinosteroids (BRs), cytokinins (CKs), and gibberellins (GAs) are classes of naturally occurring isoprenoid-derived phytohormones, which regulate plant growth and development, ranging from differentiation, through the transport of nutrients to responses on abiotic and biotic stresses (Weyers and Paterson 2001; Verma et al. 2016; Ku et al. 2018; Sytar et al. 2019). ABA is an isoprenoid sesquiterpenoid (C_{15}) synthesized from carotenoids, in most cases, from xanthophylls. The intracellular level of this compound increases when plants are exposed to biotic and abiotic stresses because ABA is a stress signaling molecule (Cutler and Krochko 1999; Li et al. 2017; Olds et al. 2018). Natural plant CKs are derivatives of adenine purine base with isoprenoid or aromatic side chain that are substituted in N^6 position. The main forms of CKs are isoprenoid free bases, including *trans*-zeatin (*t*Z) and *cis*-zeatin (*c*Z), which are the most commonly CKs occurring in many higher plants. The most abundant representatives of the aromatic side chain of CKs are *para-, meta-*, *ortho*-topolin (*p-, m-, o*T). CKs also create conjugates, most frequently with ribose or/and glucose, which are attached to the purine ring (Kieber and Schaller 2014; Hönig et al. 2018). GAs are a group of tetracyclic, diterpenoid carboxylic acids; their structure is based on *ent*-gibberellane (C_{20}) or 20-nor-*ent*-gibberellane (C_{19}) carbon skeleton (Hedden and Thomas 2012). Among more than 130 currently known GAs, just a few indicate biological activity, e.g., gibberellic acid (GA_3). Its chemical structure is a carboxylic acid skeleton with a hydroxyl group in C-3 position and a carboxyl group in C-6 position (Davière and Achard 2013). BRs are comparatively less researched class of phytohormones relative to ABA, CKs, and GAs. So far, more than 70 different BRs have been identified as free molecules or conjugates with fatty acids or glucose, and their differentiation results from the type and position of functional groups within the cyclic A and B rings and the side chain. The base of the chemical structure is the four-ring skeleton of 5α-cholestane containing ring A with hydroxyl group generally at C-2 and C-3 position, then 7-oxalactone, 6-ketone, or non-oxidized ring B and the side chain with hydroxyl group usually at C-22 and C-23. The most frequent and active types of BRs are brassinolide (BL) and castasterone (CS) (Bajguz and Tretyn 2003; Kanwar et al. 2017; Tarkowska and Strnad 2018; Zullo and Bajguz 2019).

Biological activity of phytohormones varies and depends on biosynthesis rates, cellular localization, transport, and signal perception or exposure to the biotic and abiotic stresses (Cao et al. 2016; Smith et al. 2017; Šimura et al. 2018). In recent decades, a significant increase in environmental contamination by heavy metals was observed, which causes one of the most harmful abiotic stress in the plant. Cadmium (Cd), belonging to the group of heavy metals, has a strongly toxic action for all living organisms, moreover many of aquatic, air, and soil environments are contaminated by this metal (Sytar et al. 2019). After getting inside plant cells, even a low concentration causes a toxic effect manifesting to impair life activities. Cd can form covalent and ionic bonds with biologically essential functional groups, such as sulfhydryl, amine, disulfide, carboxy, and imidazole of micro and macroelements, such as sulfur, hydrogen, oxygen, magnesium, calcium, zinc, iron, copper, and selenium (Bertin and Averbeck 2006). In plants, Cd poisoning negatively affects both physiological and biochemical cellular processes, such as photosynthesis, transpiration, and cellular respiration. In addition, Cd inhibits cell division and overall organism growth. Moreover, Cd ions present in plant cells block the activity of antioxidant enzymes located in chloroplasts and mitochondria. This causes oxidative stress and accumulation of reactive oxygen species (Liu et al. 2017).

Plant organs are a rich source of phytohormones that occur in a range of ng-fg per g of fresh weight. Thus, liquid chromatographic separation coupled with mass spectrometry (LC–MS) is the most precise method for the identification and quantification of plant hormones due to high sensitivity, accuracy, and reproducibility. A liquid chromatography quadrupole-time-of-flight mass spectrometry (LC-QToF-MS), applied in our studies, is characterized by high mass accuracy and well resolution, so it is an excellent tool for hormones profiling (Pan and Wang 2009; Bai et al. 2010; Pan et al. 2010; Xin et al. 2013; Cao et al. 2016; Chu et al. 2017; Kanwar et al. 2017; Li et al. 2019). Isolation of new or known hormones in plant species remains as a research target of many scientists. Therefore, the present study aimed to determine the endogenous level of ABA, BRs, CKs, and GAs in *W. arrhiza* using the LC-QToF-MS quantitative analysis. Moreover, the effect of different Cd concentrations (0.1–100 µM, increase by one order of magnitude) on phytohormones content was studied. Additionally, the relations between phytohormones, primarily linear, were statistically analyzed.

Materials and Methods

Growth Condition

One gram of the wild type of *W. arrhiza* was grown in sterile, glass vessels containing 200 mL of 1/30 dilution of Hunter's medium (Hutner 1953) with Cd addition in the range of concentration 0.1–100 µM except for control group. The varied solutions of metal were prepared through the diluting of $CdCl_2$ in 1/30 Hutner's medium. The breeding was grown under controlled conditions at 22.0 ± 0.5 °C, 16-h photoperiod (photon flux of 100 µmol m^{-2} s^{-1}), and 65 ± 1% humidity for 7 days. Fresh weight was harvested and filtered using a vacuum pump (KNF Laboport, Germany). Then, the sample was homogenized in liquid nitrogen using a mortar and pestle. The resulting powder was used in further analysis.

Chemicals

The standard of abscisic acid (ABA); eleven standards of BRs: 6-deoxotyphasterol (6dTY), cathasterone (CT), 6-deoxocathasterone (6dCT), typhasterone (TY), castasterone (CS), 6-deoxocastasterone (6dCS), 24-epicastasterone (ECS), brassinolide (BL), 28-norbrassinolide (28-norBL), 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) twenty-six standards of CKs: *trans*-zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), *trans*-zeatin-9-glucoside (*t*Z9G), *trans*-zeatin-7-glucoside (*t*Z7G), *trans*-zeatin-*O*-glucoside (*t*ZOG), *trans*-zeatin riboside-*O*-glucoside (*t*ZROG), *trans*-zeatin-*9*-glucoside-*O*-glucoside(*t*Z9GOG), *trans*-zeatin-9-glucoside riboside (tZ9GR), *cis*-zeatin (*c*Z), *cis*-zeatin-riboside (*c*ZR), *cis*-zeatin *O*-glucoside (*c*ZOG), *cis*-zeatin9-glucoside (*c*Z9G), *cis*-zeatin-*O*-glucoside-riboside (*c*ZROG), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), dihydrozeatin-9-glucoside (DHZ9G), dihydrozeatin-7-glucoside (DHZ7G), dihydrozeatin-*O*-glucoside (DHZOG), dihydrozeatin riboside-*O*-glucoside (DHZROG), N^6-isopentenyladenine (iP), N^6-isopentenyladenosine (iPR), N^6-isopentenyladenosine-7-glucoside (iPR7G), *para*-topolin (*p*T), *meta*-topolin (*m*T), *ortho*-topolin (*o*T), 6-benzylaminopurine (6-BAP) and standard of GA_3 were purchased from OlChemIm (Olomouc, Czech Republic). Chemicals used to prepare Hunter's medium were purchased from Sigma-Aldrich (St. Louis, USA). 4-(Dimethylamino)phenylboronic acid (DMAPBA), methanol (MeOH), acetonitrile (ACN), water (LC–MS purity), formic acid (FA) and potassium hydroxide (KOH) were purchased from Merck KGaA (Darmstadt, Germany).

Quantification of ABA, Cytokinins, and GA_3

For the measurement of phytohormones, 200 mg of plant powders were placed into the 2 mL Eppendorf tubes, suspended in 1 mL (v/v) 50% ACN and homogenized in a bead mill (50 Hz, 5 min; TissueLyser LT, Qiagen, Germany) using two 5 mm tungsten balls. Then, samples were homogenized using the ultrasound processor VCX 130 (max. power 130 W, max. frequency 20 kHz, 5 min) equipped with titanium probe (Sonics & Materials Inc., USA) and mixed in laboratory shaker (90 rpm, dark, 5 °C, 30 min; LC-350, Pol-Eko-Aparatura, Poland). Samples were centrifuged (9000×*g*, 5 min; MPW-55 Med. Instruments, Poland) and collected in a glass tube. For quantification of ABA, CKs, and GA_3, $[^2H_6]$(+)-*cis*, *trans*-ABA (50 ng), $[^2H_6]$ iP (50 ng), $[^2H_5]$ *t*Z (30 ng), $[^2H_5]$-*t*ZOG (30 ng), $[^2H_3]$-DHZR (30 ng), and $[^2H_2]$ GA_3(30 ng) were added to samples as internal standards.

Prepared extracts were purged using Waters SPE Oasis® HLB cartridge, previously activated and equilibrated using 1 mL 100% MeOH, 1 mL H_2O, and 1 mL (v/v) 50% ACN (Šimura et al. 2018). Then, extracts were loaded and collected to the Eppendorf tubes and eluted with 1 mL 30% ACN (v/v). Samples were evaporated to dryness by centrifugal vacuum concentrator (Eppendorf Concentrator Plus, Germany), dissolved in 50 µL (v/v) 30% ACN, and transferred into the insert vials. Detection of analyzed phytohormones was performed using an Agilent 1260 Infinity series HPLC system (Agilent Technologies, USA) contains QToF LC/MS mass spectrometer with Dual AJS ESI source, 10 µL of each sample was injected on the Waters XSelect C_{18} column (250 mm × 3.0 mm, 5 µm), heated up to 50 °C. Mobile phase A was 0.01% (v/v) FA in ACN and phase B 0.01% (v/v) FA in H_2O; flow was 0.5 mL min^{-1}. Separation of the above hormones was done in ESI positive mode with the following gradient: 0–8 min flowing increased linearly from 5 to 30% A, 8–25 min 80% A, 25–28 min 100% A, 28–30 min 5% A.

Quantification of Brassinosteroids

Preparation and quantification of BRs were performed as described in detail by Bajguz et al. (2019). Briefly, 200 mg of plant powders were placed into the 2 mL Eppendorf tubes, suspended in 1 mL MeOH, and homogenized using two 5 mm tungsten balls. Then, the homogenates were centrifuged, and the resulting supernatants were transferred to the flat bottom flask and mixed in laboratory shaker (90 rpm, dark, 5 °C, 90 min). For quantification of BRs, $[^2H_3]$ BL (2 ng) and $[^2H_3]$ CS (2 ng) were added into the mixture, followed by extraction with MeOH as internal standards. For screening of BRs, no internal standards were added. The samples were purified from pigments and other pollutions using Waters SPE MAX cartridge, which was activated

and equilibrated with 99.9% MeOH, H_2O, 1 M KOH, 10% (v/v) MeOH and 95% (v/v) MeOH, respectively. Purified extracts were dried up using a centrifugal vacuum concentrator, reconstructed in 10% (v/v) MeOH and passed through Waters SPE MCX cartridge for removing ion pollutions. Cartridges were previously activated and equilibrated with 5% (v/v) FA in 5% (v/v) MeOH, 5% (v/v) MeOH, 5% (v/v) NH_4OH in 5% (v/v) MeOH and 5% (v/v) MeOH, respectively. Then samples were eluted using 80% (v/v) MeOH. Eluents were dried up using a centrifugal vacuum concentrator, suspended in 96% (v/v) EtOH, and derivatized using DMAPBA reagent. Quantification of BR-DMAPBA was performed using the Agilent LC-QToF-MS system. Samples were injected on the Waters XBridge C_{18} column (250 mm × 4.6 mm, 1.7 µm); mobile phase A was 0.1% (v/v) FA in H_2O, mobile phase B was 0.1% (v/v) FA in ACN.

Optimization of MS/MS Conditions

For the optimization of MS/MS conditions, the chemical standards of analyzed phytohormones were directly injected to the MS in positive ($[M+H]^+$) ion scan modes, then areas of detected standards peaks were calculated. $[M+H]^+$ was chosen because of significantly better signal to noise ratios compared to the negative ion scan modes.

Statistical Analysis

The R software was used to perform statistical analyses R Core Team (2019). Data, grouped by phytohormone and treatment ($n=4$–5), were subjected to the one-way ANOVA ('stats' package) followed by Tukey's post hoc test ['laercio' package (da Silva 2010)]. The Shapiro–Wilk and Levene's tests ['stats' and 'car' packages] were used to verify ANOVA assumptions of Gaussian distributed data with homogenous variances ($\alpha=0.05$). Pearson's correlations ['Hmisc' package (Harrell 2019)] were calculated to check the linear relationship between each phytohormone ($n=25$), except BL, HBL, TY, 6dCS, 6dTY, *c*Z9G, *t*Z, *t*Z9G, DHZ, DHZR, DHZOG, iPR, *o*T, and *m*T, which were not normally distributed (Table 1S). Thus, all phytohormones were also assessed for a monotonic relationship using Spearman's rank correlations. Resulting correlation matrices were visualized as heatmaps using the 'corrplot' package (Wei and Simko 2017) and network plots, using the 'corrr' package (Kuhn et al. 2020), to simplify, further explore and visualize strong correlations. The projection of data on network plots was handled by multidimensional scaling of the matrix of correlations coefficients absolute values. Correlations were considered significant for $p<0.05$. Linear regression analyses were also performed to model the linear relationship between pairs of phytohormones with $|r| \geq 0.8$ ('stats' package).

Results and Discussion

Overall Phytohormones Occurrence

The presence of phytohormones has been evidenced in lower and higher plants, both in gymnosperms and angiosperms species. The most widely contributed hormone is ABA, which also occurs in cyanobacteria (Gayathri et al. 2017), marine sponges (Zocchi et al. 2001), lichens, fungi (Hirai et al. 2000; Hartung 2010), mammals (Sturla et al. 2009), and even in human blood cell (Bruzzone et al. 2007; Magnone et al. 2009) and plasma (Bruzzone et al. 2012). Other phytohormones are also commonly spread in plants. Regarding the CKs, most scientific reports present its identification and abundance within Brassicaceae family, particularly in *Arabidopsis thaliana* and *Brassica napus* (Luo et al. 2017; Šimura et al. 2018). The presence of GAs is evidenced in algae (Stirk et al. 2013a), vascular plants (MacMillan 2001; Pan et al. 2010), and fungi (Hedden and Thomas 2012). Furthermore, the distribution of the above phytohormones is reported in many genera of cyanobacteria and a few species of diatoms (Stirk et al. 2013b; Lu and Xu 2015). BRs have been identified so far in over twenty species of algae (mainly in Chlorophyceae) (Bajguz 2009; Stirk et al. 2013a; Bajguz 2019), one species of bryophyte (*Equisetum arvanse*), one pteridophyte (*Marchantia polymorpha*), two lycophytes (*Selaginella moellendorffii* and *S. uncinata*), thirteen fern species (Yokota et al. 2017), gymnosperms with genus of *Cupressus* and *Pinus*, and in angiosperms belonging to the family of Fagaceae, Apiaceae, Brassicaceae, Fabaceae, Poaceae, Solanaceae, and others (Bajguz et al. 2019; Janeczko 2019; Zullo and Bajguz 2019). Furthermore, the presence of BRs has been confirmed in all plant organs (Bajguz and Tretyn 2003; Tarkowská et al. 2016; Kanwar et al. 2017; Tarkowska and Strnad 2018). However, there are no data on the occurrence of phytohormones in the Lemnaceae family.

Endogenous Content of Phytohormones in *W. arrhiza* and Effect of Cadmium on Their Content

Quantification of phytohormones under the influence of Cd in *W. arrhiza* is presented in Table 1. Obtained results are based on the previously prepared standard curves of phytohormone content, and each value has been calculated on 1 g of fresh weight (FW). Regarding the control group, phytohormones occur in a wide range from 0.016 to 55.541 ng g^{-1} FW. Thus, the LC-QToF-MS analysis indicated the presence of ABA, eight BRs (CT, TY, 6dTY, CS, 6dCS, ECS, BL, and HBL), five isoprenoid free bases

Table 1 The phytohormones level (ng g^{-1} fresh weight) in *Wolffia arrhiza* treated with Cd

	Control	0.1 µM Cd	1 µM Cd	10 µM Cd	100 µM Cd
ABA	0.348 ± 0.128^{c}	0.448 ± 0.071^{c}	0.532 ± 0.075^{bc}	0.7 ± 0.05^{b}	1.011 ± 0.141^{a}
GA_3	55.541 ± 4.028^{a}	47.951 ± 2.011^{b}	43.464 ± 3.28^{b}	32.443 ± 2.322^{c}	25.136 ± 2.247^{c}
6dTY	0.897 ± 0.081^{b}	1.061 ± 0.141^{b}	0.998 ± 0.086^{b}	1.559 ± 0.159^{a}	0.774 ± 0.114^{b}
CT	1.709 ± 0.437^{c}	3.55 ± 0.293^{c}	7.006 ± 0.73^{a}	6.621 ± 0.47^{a}	5.062 ± 0.4^{b}
TY	0.351 ± 0.033^{b}	0.348 ± 0.027^{b}	0.531 ± 0.027^{b}	0.913 ± 0.156^{a}	0.514 ± 0.077^{b}
6dCS	0.106 ± 0.032^{c}	0.135 ± 0.006^{c}	0.177 ± 0.016^{c}	0.343 ± 0.037^{b}	0.807 ± 0.037^{a}
CS	3.821 ± 0.184^{abc}	4.004 ± 0.201^{ab}	4.568 ± 0.27^{a}	3.698 ± 0.241^{bc}	3.133 ± 0.606^{c}
ECS	1.067 ± 0.086^{a}	1.024 ± 0.11^{a}	0.72 ± 0.124^{b}	0.524 ± 0.095^{b}	0.52 ± 0.043^{b}
BL	0.179 ± 0.027^{b}	0.174 ± 0.017^{b}	0.264 ± 0.132^{b}	0.464 ± 0.109^{b}	1.697 ± 0.19^{a}
HBL	1.401 ± 0.315^{c}	1.356 ± 0.093^{c}	1.744 ± 0.227^{bc}	2.342 ± 0.048^{b}	3.656 ± 0.348^{a}
*t*Z	23.235 ± 1.7^{b}	22.614 ± 1.931^{b}	33.619 ± 3.194^{a}	24.577 ± 1.283^{b}	5.261 ± 0.751^{c}
*t*ZR	7.332 ± 1.588^{c}	17.285 ± 2.684^{b}	27.36 ± 2.94^{a}	13.876 ± 1.92^{b}	2.775 ± 0.776^{c}
*t*Z9G	0.185 ± 0.091^{a}	0.232 ± 0.045^{a}	0.102 ± 0.015^{a}	0.341 ± 0.491^{a}	0.068 ± 0.013^{a}
*t*Z7G	0.03 ± 0.006^{bc}	0.032 ± 0.002^{bc}	0.038 ± 0.009^{ab}	0.046 ± 0.003^{a}	0.02 ± 0.005^{c}
*t*ZROG	0.205 ± 0.035^{c}	0.283 ± 0.096^{bc}	0.52 ± 0.088^{a}	0.432 ± 0.108^{ab}	0.449 ± 0.046^{a}
*c*Z	20.52 ± 1.769^{a}	15.918 ± 1.839^{b}	11.306 ± 0.843^{c}	9.357 ± 0.635^{c}	2.236 ± 0.178^{c}
*c*Z9G	0.027 ± 0.004^{c}	0.605 ± 0.116^{a}	0.692 ± 0.118^{a}	0.549 ± 0.088^{ab}	0.386 ± 0.071^{b}
DHZ	0.071 ± 0.009^{c}	0.338 ± 0.013^{b}	0.479 ± 0.089^{a}	0.27 ± 0.01^{b}	0.049 ± 0.013^{c}
DHZR	2.518 ± 0.395^{c}	5.682 ± 0.667^{b}	7.469 ± 0.852^{a}	4.077 ± 0.762^{c}	3.036 ± 0.817^{cc}
DHZOG	4.683 ± 0.838^{c}	15.429 ± 0.919^{a}	12.726 ± 1.264^{b}	8.28 ± 0.777^{c}	4.479 ± 1.073^{c}
iP	0.375 ± 0.105^{a}	0.292 ± 0.075^{a}	0.434 ± 0.1^{a}	0.33 ± 0.046^{a}	0.048 ± 0.01^{b}
iPR	0.083 ± 0.017^{c}	0.222 ± 0.057^{c}	0.405 ± 0.035^{c}	1.064 ± 0.166^{a}	0.722 ± 0.104^{b}
iPR7G	0.383 ± 0.109^{c}	2.662 ± 0.442^{b}	3.474 ± 0.362^{a}	2.038 ± 0.634^{bc}	1.393 ± 0.227^{c}
*o*T	0.287 ± 0.041^{c}	0.509 ± 0.072^{c}	0.586 ± 0.038^{bc}	0.71 ± 0.044^{b}	1.563 ± 0.104^{a}
*m*T	0.016 ± 0.002^{c}	0.078 ± 0.014^{bc}	0.142 ± 0.012^{bc}	0.187 ± 0.017^{b}	0.81 ± 0.139^{a}

Data, grouped by treatment for each phytohormone, represent the mean ($n = 4$–5) ± standard deviation. The same letters indicate statistically nonsignificant differences, according to Tukey's post hoc test ($p \geq 0.05$)

of CKs (*t*Z, *c*Z, DHZ, iP, and iPR), two aromatic free bases of CKs (*o*T and *m*T), eight conjugates of CKs (*t*ZR, *t*Z9G, *t*Z7G, *t*ZROG, *c*Z9G, DHZR, DHZOG, and iPR7G), and GA_3. The total number of detected compounds is 25. This is the first report about the presence of plant hormones not only in *W. arrhiza* but also in Lemnaceae plants. This study showed that the most widely contributed phytohormones are CKs, which are represented by 15 compounds. Among all detected phytohormones, the highest content of GA_3 and *t*Z was noted (55.541 and 23.235 ng g^{-1} FW, respectively). The total level of hormone groups in duckweed exposed to Cd is presented in Fig. 1. Therefore, the total content of ABA and BRs increased while GA_3 amount decreased in the presence of Cd. While the level of CKs in plant exposed to 0.1, 1, and 10 µM Cd was higher than control, but in plant treated of 100 µM Cd was lower.

The correlation analysis of phytohormones in *W. arrhiza* revealed very strong, negative linear relations between ABA vs. GA_3, ECS, and *c*Z; GA_3 vs. iPR; and ECS vs. iPR [according to Evans (1996)] (Fig. 2a, b), while positive was found between GA_3 vs. ECS and *c*Z; ECS vs. *c*Z; CT vs. *t*ZROG; and *t*ZR vs. iPR7G. Ten linear regressions models and equations were calculated and presented for those pairs of phytohormones (Fig. 2a), e.g., ABA ≈ 1.404 − 0.019 × GA_3. Thus, the precise relationship was identified in this research. Furthermore, strong, negative monotonic relations were found between, e.g., *c*Z vs. *o*T, *m*T, iPR, 6dCS, IAA, BL, and HBL; HBL vs. GA_3 and ECS; IAA vs. GA_3; ECS vs. 6dCS. Positive ones were noted between, e.g., BL vs. iPR, *o*T, *m*T, IAA, ABA, 6dCS, and HBL; TY vs. iPR, *m*T, IAA, 6dCS, and HBL; DHZR vs. *c*Z9G, DHZ, DHZOG, iPR7G, *t*ZR, IPA, and CS (Fig. 3a, b).

ABA, as an essential hormone during heavy metal stress, is synthesized through the methylerythritol phosphate (MEP) or methylerythritol phosphate (MVA) pathway. Their precursor is isopentenyl pyrophosphate (IPP), which is synthesized in higher plants through MEP in the cytosol and MVA in the plastid, whereas in algae exclusively through MEP in the cytosol. Transformations of IPP led to the origin of xanthophyll, which are direct precursors of ABA (Maršálek and Šimek 1992; Cutler and Krochko 1999; Li et al. 2017; Olds et al. 2018). Biosynthesis of ABA significantly increases under stress conditions, e.g., salinity, drought, cold temperature, or heavy metals (Khan et al. 2020), therefore the

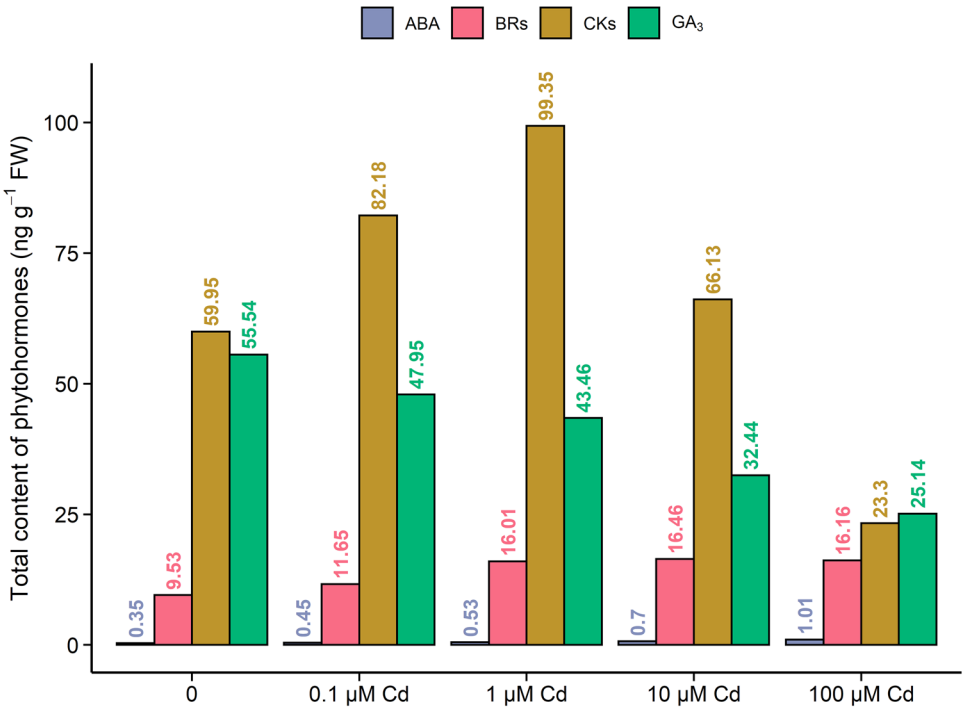

Fig. 1 The total content of phytohormones (ng g^{-1} fresh weight) in *W. arrhiza* treated with cadmium

endogenous level of ABA enhanced under the influence of Cd, reached the highest value for 100 µM Cd (1.011 ng g^{-1} FW, Table 1, Fig. 1) in *W. arrhiza*. Thus, this is almost a threefold increase comparing to untreated duckweed.

Biosynthesis of BRs is a multistep process, including three independent pathways for creating C_{27}, C_{28}, and C_{29} types of BRs. During this research, compounds belonging to the C_{28} type were identified, except HBL, which has 29 atoms of carbon. Synthesis of C_{28} BRs can occur in both early and late oxidation pathway from campestanol (CN), which is a direct precursor of this BRs biosynthesis type. During the early C6 oxidation pathway, CN is hydroxylated in C-6 position to 6α-hydroxycampesterol, which is oxidized to 6-oxocampestanol (6-oxoCN). It is hydroxylated in C-22 position to the first of BR – CT. Next, CT is hydroxylated in C-23 position to TE, which is converted in 3-dehydroteasterone, and this BR is reduced in C-3 position to TY. Then, TY is hydroxylated in C-2 position to CS, which is oxidized in C-7 position to BL. Whereas in the late C_6 oxidation pathway, CN is converted to 6dCT, which is hydroxylated to 6dTE, the next 6dTE is reduced to 3-dehydro-6dTE and this compound is hydroxylated to 6dTY which is hydroxylated in C-2 position to 6dCS. Next, 6dCS after a hydroxylation to 6-hydroxyCS is oxidized in C-6 position to CS, which is oxidized to BL (Wang et al. 2017; Ohnishi 2018). In the present study, the presence of hydroxylated and no hydroxylated forms of BRs was reported. The occurrence of TY (a direct precursor of CS biosynthesis in the early C_6 pathway) and 6dCS (a precursor of CS during the late C6 pathway) shows that the biosynthesis of BRs in *W. arrhiza* can occur in both pathways. The previous study of Bajguz and Asami (2005) indicates that the addition of brassinazole (a specific BR biosynthesis inhibitor) to *W. arrhiza* cultures inhibits their growth, which was reversed by exogenous EBL. Brassinazole blocks the conversion of CN to 6dCT, 6dCT to 6dTE, 6-oxoCN to CT, and CT to TE (Asami and Yoshida 1999; Rozhon et al. 2019). It confirms that BRs are essential to the normal growth of *W. arrhiza*. In this study, among untreated with Cd plants, the largest content of CS and CT was noted (3.821 and 1.709 ng g^{-1} FW, respectively). Sitosterol, as a precursor of C_{29} biosynthesis, is transformed into 28-homoTY, which then is converted to 28-homoCS and HBL (Roh et al. 2017). The presence of HBL in *W. arrhiza* suggests the occurrences of the C_{29} biosynthesis pathway in this duckweed. Whereas C_{27} type of BR, i.e., 28-norBL has not been detected. However, many C_{27} compounds, e.g., 28-norCT, 28-norTE, 28-norTY, 28-norCS, have not been noted. Thus, the presence of the C_{27} pathway cannot be excluded. Differences between amount and distribution of various types of BRs are related with family, e.g., Brassicaceae, Poaceae, or Solanaceae (Bajguz and Tretyn 2003; Verhoef et al. 2013; Xin et al. 2013; Tarkowská et al. 2016; Kanwar et al. 2017; Tarkowska and Strnad 2018; Bajguz et al. 2019; Janeczko 2019; Li et al. 2019). Exposure of *W. arrhiza* culture on Cd caused an increase of the endogenous level of BRs in relation to control (Table 1), except ECS, whose amount decreased. The level of 6dCS, BL, and HBL increased proportionally to the rising of Cd concentration. In the case of 6dTY and TY the largest value noticed in plant treated with 10 µM Cd, while the level of CT and CS was

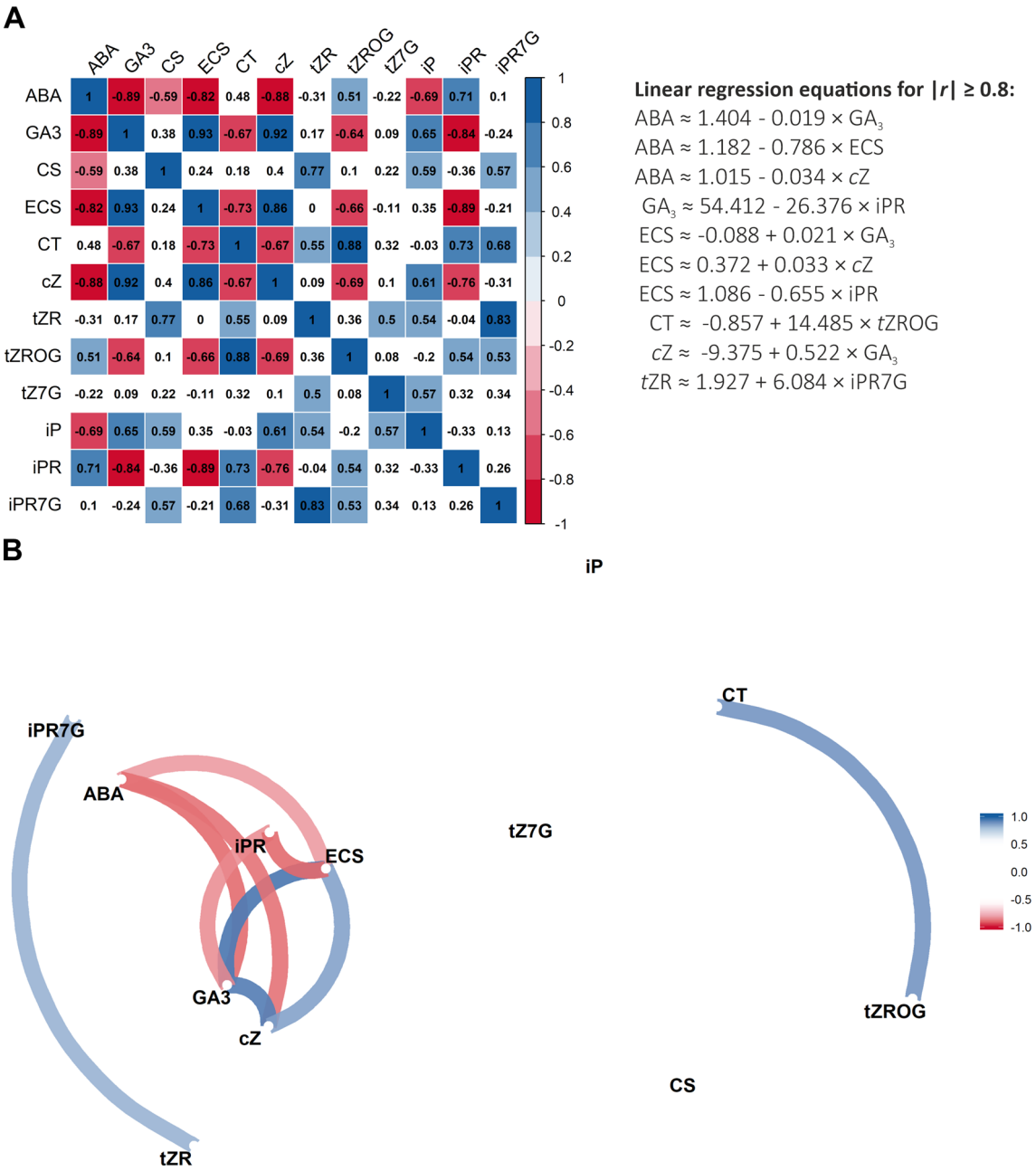

Fig. 2 **a** Correlation heatmap for phytohormones ($n = 22$) in *Wolffia arrhiza* with equations obtained from linear regression. The colored and labeled scale codes for the value of Pearson's correlation coefficient *r*. Positive correlations are blue, while negative correlations are red. White squares show nonsignificant correlations ($p \geq 0.05$). **b** Multidimensional scaling network plot of the absolute values of $r \geq 0.8$ (Color figure online)

the highest in 1 µM Cd. Bali et al. (2019) show the positive effect of exogenously applied BRs on Cd treated plant, e.g., HBL increases the activity of antioxidants and overcomes the inhibition of plant growth, but there are no data about the endogenous level of BRs in plants exposed to Cd. However, increased biosynthesis of BRs in duckweed exposed to Cd (Fig. 1) confirmed the role of BRs in the response of plants to the heavy metal stress.

In lower plants (mosses, green algae, ferns, horsetails), CKs have been only identified as free bases of *c*Z and iPR; and their riboside conjugates, whereas in higher plants occurrence of all currently known free bases types and conjugates of CKs was reported (Stirk and van Staden 2003; Bajguz and Piotrowska 2009; Aremu et al. 2012). Biosynthesis of CK isoprenoid occurs through the transfer of C_5 isoprenoid unit to adenine molecule that may be a free

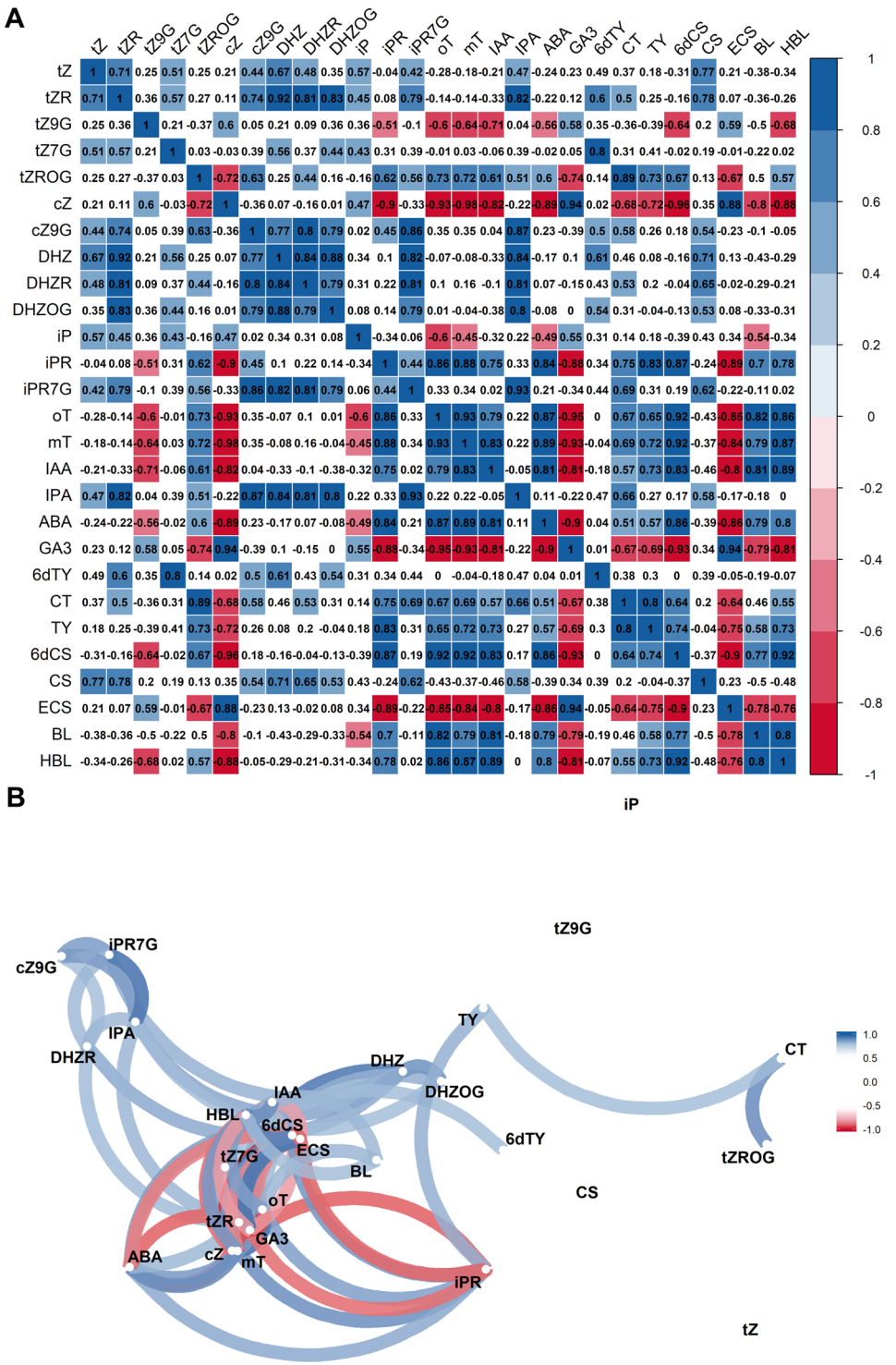

Fig. 3 **a** Correlation heatmap for phytohormones ($n = 22$) in *Wolffia arrhiza*. The colored and labeled scale codes for the value of the Spearman's rank correlation coefficient r_s. Positive correlations are blue, while negative correlations are red. White squares show nonsignificant correlations ($p \geq 0.05$). **b** Multidimensional scaling network plot of the absolute values of $r_s \geq 0.8$ (Color figure online)

nucleotide (AMP, ADP, or ATP) or bound with tRNA. There are two donors of C_5 isoprenoid, first of them is dimethylallyl pyrophosphate that is synthesized during a MEP or MVA pathway, second of them is 4-hydroxy-3-methyl-2-(E)-butenyl diphosphate that is formed only by MEP. These reactions are catalyzed by adenylate isopentenyl transferases. Then, obtained compounds are hydroxylated to *tZ* by cytochrome P450 monooxygenase. CKs are also produced by the degradation of tRNA, and this is the main source of CKs isoprenoids forms in *cis* configuration (Frébort et al. 2011; Kieber and Schaller 2014; Feng et al. 2017; Tarkowska and Strnad 2018). Our results indicate the presence of fifteen forms of CKs and show the effect of Cd application on their content (Table 1, Fig. 1). Regarding the control, the results include

44.284 ng g^{-1} FW of isoprenoid free bases, 0.303 ng g^{-1} FW of aromatic free bases, and 15.363 ng g^{-1} FW conjugates of CKs in *W. arrhiza*. Furthermore, the presence of CKs either in *cis* or *trans* orientations was reported. Among all detected CKs, the highest content of *t*Z, and *c*Z was noted (23.235 and 20.52 ng g^{-1} FW, respectively). While due to the chemical form of CKs; *t*Z, *c*Z-types are the predominant (30.987 and 20.547 ng g^{-1} FW, respectively), DHZ-type occurs in less amount (7.272 ng g^{-1} FW) and iP-type of CKs presents in low amount, i.e., 0.841 ng g^{-1} FW. Generally, the endogenous level of CKs after application of Cd was higher than in control; however, a concentration of 100 µM Cd for several compounds caused inhibition of their synthesis. For example, the amount of *t*Z under the influence of 0.1 and 10 µM Cd was similar to the control, in the plant treated 1 µM Cd significantly increased, while in 100 µM of Cd decreased. Merely content of *c*Z was reduced in all concentrations of Cd compared to the control. However, the overall content of CKs in duckweed exposed to the 0.1–10 µM Cd was larger to untreated plant, but the application of 100 µM Cd caused a considerable decline of CKs level (Fig. 1, Table 1). Zhou et al. (2019) also demonstrated a slight increase of total CKs concentration in *Kosteletzkya pentacarpos* seedlings in the presence of 10 µM Cd to control. Interestingly, they indicated a positive effect of exogenously applied *t*ZR on plant treated with 10 µM Cd, which can explain the enhanced biosynthesis of *t*ZR in present results. The application of 100 µM Cd also caused a decrease of Z and ZR levels in soybean (Hashem 2014) and *W. arrhiza* (Table 1). The percentage content of types of CKs in duckweed with the addition of Cd is presented in Fig. 4. Free bases of CKs are the most widespread in *W. arrhiza,* but in a group with Cd their predominance over conjugates is lower comparing to the control group. Consequently, an increased proportion of all CKs conjugates to free bases in plants treated with Cd was noted. The percentage of *O*-glucoside forms increased from 16.57% in control up to about 30% in exposure to Cd plant. The contribution of riboside and *N*-glucosides conjugates also was enhanced. Despite the conviction that *t*Z and iP forms are the dominant types of CKs; there are reports about the dominance of *c*Z in many plants, e.g., in potatoes, rice, maize, and legumes (Gajdošová et al. 2011; Murai 2014; Schäfer et al. 2015). In the current research, *t*Z is a basic and most common form of CKs; however, the occurrence of iP form is limited. The primary function of CKs is the stimulation of cell division and the prevention of cells aging (Sosnowski et al. 2019). Moreover, Kurepa et al. (2018) reported the positive correlation between exogenous applied of 6-benzyladenine and increases of cell size and division in two species of duckweeds (*Spirodela polyrhiza* and *Lemna gibba*). It confirms the importance of CKs in both grown and development of Lemnaceae plants.

In higher plants, the most frequently active forms of GAs are GA_1, GA_3, and GA_4. The first step of GAs biosynthesis is a transformation of geranylgeranyl diphosphate to the *ent*-kaurene in the plastid, then conversion of *ent*-kaurene to the

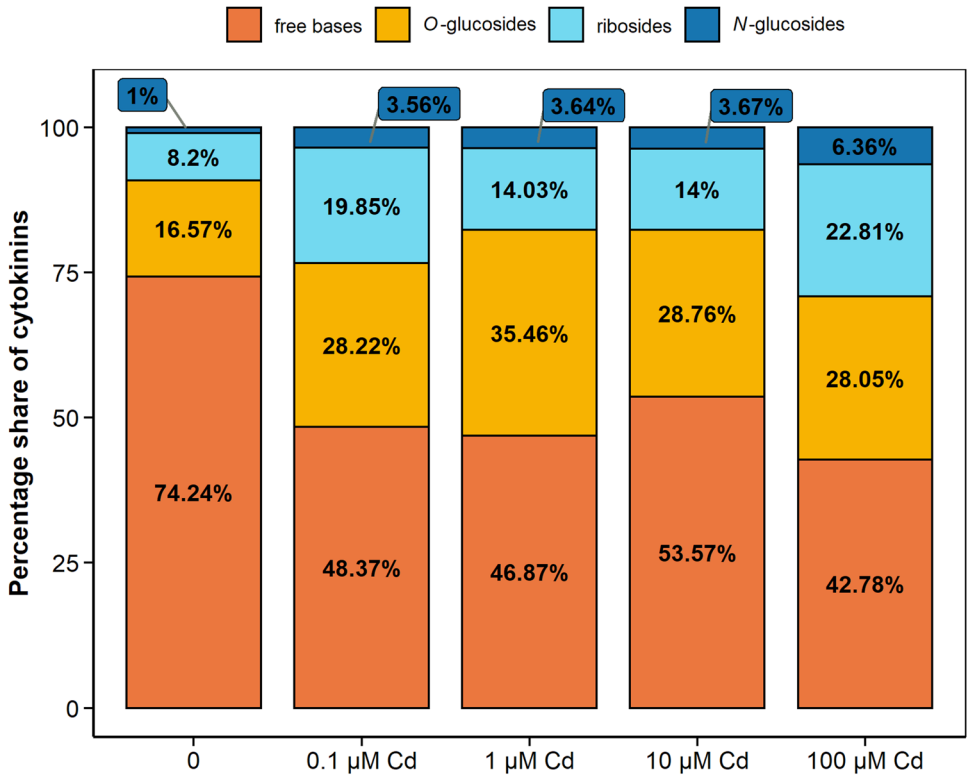

Fig. 4 The percentage share of different types of cytokinins: free bases (*t*Z, *c*Z, DHZ, iP, *o*T, *m*T) and conjugates: *N*-glucosides (*t*Z9G, *t*Z7G, iPR7G), *O*-glucosides (*t*ZROG, *c*Z9G, DHZOG), ribosides (*t*ZR, DHZR, iPR) in *Wolffia arrhiza* treated with cadmium

various intermediates until the synthesis of GA_{12} aldehyde in the endoplasmic reticulum, finally a synthesis of GA_3 from GA_{12} in the cytosol. Several *ent*-kaurene oxidation steps lead to the formation of GA_{12} aldehyde, whereas the formation of GA_{12} occurs through oxidation of their aldehyde group to the carboxyl group. Further, the conversions of GA_{12} led to the active synthesis form of GAs, i.e., GA_3 (Kasahara et al. 2002; Hedden and Thomas 2012; Gao et al. 2017). The largest content of GA_3 (55.541 ng g^{-1} FW) among all identified phytohormones in *W. arrhiza* was noted (Table 1). It confirms the finding of Pieterse (1974) that the absence of flowers in duckweed relates to the high level of endogenous GA_3. In this study, the flowering of *W. arrhiza* has not been observed because it is a tropical and subtropical flowering plant. It is thus surprising that the flowering of *W. arrhiza* was discovered for the first time in Central Europe in Germany (Schmitz and Kelm 2017). Therefore, future studies should address the induction of flowering by creating optimal conditions in plant growth cabinets. It is commonly known that phytohormones contribute to the flowering process (Conti 2017); hence, the level of hormones will be examined in *W. arrhiza*. While in the present analysis, the decline of GA_3 level proportionally to the increase of Cd concentration (Fig. 1) was reported. Moreover, recent studies of Zhou et al. (2019) indicated that 10 µM Cd treatment reduced GAs content in *Kosteletzkya pentacarpos* seedlings. Obtained results suggest the negative effect of Cd stress on GAs. Atici et al. (2005) showed a decrease of GA_3 content in chickpea seeds treated with lead. All the mentioned results suggest the negative effect of Cd or other heavy metals stress on biosynthesis and endogenous level of GAs.

Conclusion

In this work, the presence of endogenous isoprenoid-derived phytohormones, and the effect of Cd on their content is reported for the first time in *W. arrhiza*. The total number of detected compounds is 25, and they belong to four groups of phytohormones, i.e., ABA, BRs (eight compounds), CKs (15 compounds), and GAs (one compound). The content of phytohormones, especially BRs, was changed in Cd dose-dependent manner. Treatment with Cd causes an increase in the content of ABA, BRs, and CKs (except 100 µM Cd). Simultaneously, the content of GA_3 was inversely proportional to the increasing Cd concentration. Overall, the distribution of ungrouped data showed linear and monotonic dependencies between phytohormones.

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Author contributions Concept of the study: AB. Analysis and interpretation of MS data: all authors. Preparing a draft of the manuscript: MC. Final approval of manuscript: all authors.

Compliance with Ethical Standards

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

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