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The response of a model C_3/CAM intermediate semi-halophyte *Mesembryanthemum crystallinum* L. to elevated cadmium concentrations

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ABSTRACT

Many areas exhibiting increased concentrations of soluble salts are simultaneously polluted with heavy metals (HM), and halophytes with extended tolerance to heavy metal toxicity seem to represent a promising tool for their phytoremediation. In this study, the response of the soil-grown C_3-CAM (Crassulacean acid metabolism) intermediate halophyte *Mesembryanthemum crystallinum* (common ice plant) to increased concentrations of Cd (0.01–1 mM) was investigated. None of the tested Cd treatments affected growth parameters or tissue water content of either C_3 or CAM-performing plants. Chlorophyll *a* fluorescence confirmed high tolerance of the photosynthetic apparatus of both metabolic states towards Cd. Plants performing both photosynthesis types accumulated significant Cd amounts only under the highest (1 mM) treatment, and the metal was primarily deposited in the roots, which are features typical of an excluding strategy. Upon the application of 1 mM Cd solution CAM-performing plants, due to the NaCl pre-treatment applied for CAM induction, were exposed to significantly higher amounts of bioavailable Cd in comparison with those of C_3-performing plants. As a result, roots of CAM plants accumulated over 4-fold higher Cd amounts when compared with C_3 plants. In our opinion, enhanced Cd-accumulating potential observed in CAM-performing plants was the effect of osmotic stress episode and resulting modifications *e.g.* in the detoxifying capacity of the antioxidative system. Increased antioxidative potential of NaCl pre-treated plants was pronounced with significantly higher activity of CuZnSOD (copper-zinc superoxide dismutase), not achievable in C_3 plants subjected to high Cd concentrations. Moreover, the applied Cd doses induced SOD activity in a compartment-dependent manner only in C_3 plants. We confirmed that none of the applied Cd concentrations initiated the metabolic shift from C_3 to CAM.

1. Introduction

Heavy metal (HM) contamination is a serious problem that has rapidly increased over the past decades, primarily as a result of human activities such as mining and extensive agriculture. Exposure to different types of HM pollution is a concern for all plants and in consequence, may affect primary trophic chains, including those with humans (Shaw et al., 2004; Nagajyoti et al., 2010; Maleki et al., 2017). Cadmium is one of the most hazardous and toxic heavy metal that occurs in nature in relatively small amounts but can be at high concentrations in soils, waters, and air predominately because of anthropogenic activities. In addition to various industrial processes, the primary sources of Cd pollution are fertilizers commonly used in agriculture. The high genotoxicity and ecotoxicity of Cd towards plants are well described in earlier studies (Shevyakova et al., 2003; Benavides et al., 2005; Pavlaki et al., 2016). Growth inhibition, the most visible effect of cadmium toxicity in sensitive plants, results primarily from the influence of heavy metals on stomata opening, photosynthesis, respiration and uptake of essential elements such as Ca, Mg, P, K and N (Das et al., 1997; Pagliano et al., 2006; Shukla et al., 2008). At least part

Abbreviations: CAM, Crassulacean acid metabolism; CuZnSOD, copper-zinc superoxide dismutase; Fv/Fm, maximum quantum efficiency of photosystem II; GF-AAS, Graphite Furnace-Atomic Absorption Spectroscopy; HM, heavy metal; ICP-OES, Inductively Coupled Plasma - Optical Emission Spectrometry; IRT, iron-regulated transporter; PI, performance index; SOD, superoxide dismutase; TWC, tissue water content; ZIP, ZRT IRT-like Protein; ZRT, zinc-regulated protein

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Table 1
The composition of bioavailable macro- and micronutrients of the soil substrate.

	NH_4	NO_3	P	K	Fe	Na	Mg	S	Cu	Zn	Mn
Concentration [mg kg^{-1}]	21 ± 5	29 ± 7	18 ± 2	883 ± 119	1 ± 0.1	238 ± 54	423 ± 33	274 ± 62	2 ± 0.3	2 ± 0.4	5 ± 1

(N = 4, mean value ± SD).

of these effects develops because of metal-induced redox oxidative stress and oxidative damage in which the overproduction of reactive oxygen species (ROS) is initiated by the increased activity of NADPH oxidases modified with exposure to Cd (Heyno et al., 2008). The many generated superoxide radicals ($O_2^{\cdot -}$) together with the resultant redox imbalance require a fast and efficient cell response, involving in particular, intensified detoxification with superoxide dismutases (SOD) (Halliwell and Gutteridge, 2015).

Cadmium and other HMs cannot be subjected to biodegradation and therefore must be removed from the contaminated ground and water environments with physical and/or chemical techniques. One of the practical approaches is the use of plants in the process of phytoextraction, which currently is the most commercially applicable method. However, in many areas polluted with HM, high concentrations of soluble salts occur concomitantly (Lutts and Lefèvre, 2015). Halophytes seem to be suitable natural candidates for phytoremediation of such areas, not only due to increased tolerance to salinity. Recent studies suggest that halophytes also represent enhanced ability to cope with heavy metal stress (HMS) (Ghnaya et al., 2005; Ali et al., 2013; Amari et al., 2014). Halophytes simultaneous resistance to HMS and high salts concentrations is, at least partially, attributed to common physiological processes (Manousaki and Kalogerakis, 2011).

The common ice plant (*Mesembryanthemum crystallinum*) is a well-recognized halophyte which originates from dry and hot areas of the Namib Desert (South Africa). In a result, it is resistant to numerous environmental stresses including drought, salinity and high light intensity (Adams et al., 1998). While most plants exhibit C_3 photosynthesis, the Crassulacean acid metabolism (CAM), often described as a water-saving pathway, is performed by plants with frequently limited water access (Borland et al., 2006). The common ice plant is a well-known C_3-CAM intermediate, and high plasticity of its photosynthetic metabolism, understood as the ability to shift between C_3 and CAM type, was confirmed (Winter and Holtum, 2014; Nosek et al., 2018). In practice, well-watered ice plants exhibit C_3 photosynthesis and upon introduction of osmotic stresses initiate CAM (Cushman et al., 1990; Hurst et al., 2004; Winter et al., 2008). Beside enhanced tolerance to salinity and plasticity of photosynthetic metabolism, the common ice plant's resistance to high concentrations of Zn, Cu and Ni have been confirmed (Thomas et al., 1998; Kholodova et al., 2005; Amari et al., 2014). Kuznetsov et al. (2000) demonstrated that *M. crystallinum* can grow and function under high concentrations of NaCl (up to 800 mM) and Cu (up to 8 mM) in the soil. Presented data indicate that a common ice plant is a convenient object not only for studies concerning phytoremediation of high salinity areas but also surveys regarding the relationship between plant photosynthesis and phytoremediation potential.

The primary aims of this investigation were to 1) study the response of soil-grown *M. crystallinum* plants to increased concentrations of Cd, 2) determine the potential of plants exhibiting both metabolic types for Cd accumulation in roots and shoots, 3) characterize the response of SOD, the primary enzymatic antioxidant, to increased heavy metal quantities in terms of performed photosynthesis type, and 4) verify whether, similarly to other abiotic stressors (excess light, drought, salinity), elevated Cd levels can induce the CAM in C_3-performing plants. We suspect that different responses of C_3- and CAM-performing ice plants due to differences in their morphology, anatomy, and physiology would be reflected in different accumulating properties.

2. Materials and methods

2.1. Plant cultivation and Cd treatment

Mesembryanthemum crystallinum L. plants seeds were sown on soil substrate in a cultivation chamber under 250–300 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), a 16/8 h day/night period (25/17 °C temperature) and 65/70% relative humidity (RH). Two weeks after sowing, each seedling with a fully developed 2nd leaf pair was transferred to an individual pot with dimensions 95 × 90 x 90 mm. The substrate used in each experimental stages was made of market available soil ("Athena" Bio-Products, Golczewo, Poland; pH 6.75; d = 0.24 kg dm^{-3}) and sand (grain size in the range of 1–2 mm) mixed in 4:1 v/v ratio; substrate mass applied per individual pot was 360 ± 0.1 g. The concentrations of bioavailable macro- and micronutrients measured in the soil substrate has been reported in Table 1. After 6 weeks, the plants were divided into two groups: the first group was irrigated with tap water (C_3 plants) and the second group with 0.4 M NaCl to induce CAM (CAM plants). After 14 days, CAM was confirmed in the NaCl pre-treated plants with measurement of the diurnal Δ malate (the difference between malate concentration measured at the beginning and the end of the light phase) in the leaf cell sap in accordance with the spectrophotometric protocol described earlier in the work of Gawronska and Niewiadomska (2015). In the next step, 8-week-old C_3 and CAM plants each day of the 8-day long treatment were irrigated with 10 cm^3 solution of 0 (control), 0.01, 0.1 and 1 mM $CdCl_2$ (Sigma Aldrich, USA). Applied Cd solutions for C_3- and CAM-performing plants were based on water and 0.4 M NaCl, respectively. No leakage from pots was detected during Cd application. The day after the Cd treatment, following completion of fluorometric analyses, four plants of each experimental variant were harvested (roots and shoots independently). The 4th pair of leaves of each plant was collected. One leaf from this pair was gathered into a pooled sample, immediately frozen in liquid nitrogen, ground and then stored at −80 °C for protein activity assay, while the second leaf was collected for ^{13}C isotope determination. The remaining shoots (including leaves) and roots were used for biometric and Cd concentration analyses.

2.2. Plant growth parameters

For growth parameter and tissue water content determination, plant organs were prepared according to the procedures described in Amari et al. (2014), with slight modification regarding desiccation temperature. Namely, collected root parts were gently rinsed with cold distilled water until soil substrate was removed; shoots were rinsed briefly and together with roots were blotted with filter papers. For biometric analyses, the fresh weight was measured immediately, and the dry weight was assessed after 48 h of desiccation in an oven at 105 °C. The tissue water content (TWC) was calculated on a dry weight basis as:

$$TWC\ (cm^3\ g^{-1}\ DW) = (FW - DW)/DW$$

where FW and DW are the fresh and dry weights, respectively.

2.3. Chlorophyll a fluorescence

Chlorophyll fluorescence measurements were performed with a Handy Pea chlorophyll fluorometer (Hansatech Instruments, UK) at the

light phase's beginning the day after the last Cd treatment was applied. Before the measurement, two intact mature leaves of each plant (9 weeks old) were dark-adapted for 20 min with clips supplied by a manufacturer.

The maximum quantum efficiency of PSII, QY_{max}, was calculated using the following equation (Genty et al., 1989):

$$QY_{max} = Fv / Fm$$

where Fv and Fm stand for the variable and maximum fluorescence, respectively.

To assess plant vitality, the performance index (PI_{total}) was calculated with a multi-parametric indicator (Strasser et al., 2000):

$$PI_{total} = PI_{ABS} \times \frac{RE}{ABS}$$

where PI_{ABS} is the performance index on an absorption basis and $\frac{RE}{ABS}$ indicates the contribution of the reduction of end equivalents.

2.4. Analysis of soil substrate's bioavailable macro- and micronutrients composition

For determination of macro- and micronutrients composition, untreated soil substrate was dried for 2 weeks at room temperature and sieved through a 1 mm diameter sieve. Extraction of bioavailable macro- and micronutrients was performed according to the method described by Ostrowska et al. (1991); substrate sample was washed for 30 min with 200 cm^3 of 30 mM CH_3COOH solution. After extraction, the quantities of macro- and micronutrients was determined using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Prodigy Teledyne Leeman Labs, USA).

2.5. Cadmium concentration analysis

The soil substrate remaining after plant harvest was collected and stored at 4 °C. Prior to the analysis, the soil substrate was dried for 48 h in an oven at 105 °C and sieved (1 mm). Measurements of soil Cd content were performed according to the Rinkis method with the extraction of 1 mol dm^{-3} HCl as previously described by Ostrowska et al. (1991). This technique employs a relatively "aggressive extractant" enabling the extraction of more than only plant available forms of elements including exchangeable and weakly adsorbed fractions of ions. After extraction, the quantity of Cd was determined using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Prodigy Teledyne Leeman Labs, USA).

For determination of Cd concentrations in root and shoots of *M. crystallinum*, the method employing Graphite Furnace-Atomic Absorption Spectroscopy (GF-AAS, Thermo iCE3000, USA) was applied, as previously described by Rozpądek et al. (2018). All standards were purchased from Sigma Aldrich (Germany).

2.6. Carbon isotope analysis

For determination of ^{13}C ratio leaves of 4th pair were additionally oven-dried for 24 h at 105 °C and ground to a fine powder. Isotope ratio measurements of (discrimination) were performed on a Finnigan MAT 253 Mass Spectrometer (ThermoFinnigan, Germany) coupled with a Flash HT Elemental Analyser (Thermo Scientific, USA) operating at a continuous flow mode.

2.7. Protein extraction and quantification

To analyse superoxide dismutase (SOD), crude protein was extracted in accordance with the procedure described by Libik-Konieczny et al. (2012). Briefly, 1 g of frozen leaf tissue was homogenized in a buffer containing 100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT (dithiothreitol), and 3 mM EDTA, adjusted with 1 M Tris to pH 8.0. The homogenate was centrifuged at 12,000 *g* at 4 °C for 5 min. The protein content in the supernatant was determined by the Bradford (1976) assay, with bovine serum albumin (BSA) as the standard.

2.8. Analysis of superoxide dismutase (SOD) by native polyacrylamide gel electrophoresis (native-PAGE)

Electrophoretic separation of soluble protein fractions was performed according to Laemmli (1970); native-PAGE was conducted at 4 °C and 180 V on discontinuous 12% polyacrylamide gels. For visualization of SOD bands, gels were incubated in the staining buffer pH 7.8 containing 50 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 0.25 mM NBT, 20 µM riboflavin, 2.5 mM TEMED in darkness at room temperature for 20 min and then exposed to white light until SOD activity bands became visible (Beauchamp and Fridovich, 1971). SOD bands were analysed densitometrically with ImageJ 2 (GPL license).

2.9. Statistical analysis

The results were analysed with Statistica 13.3 (StatSoft, USA) statistical software. One-way ANOVA followed by post-hoc test was used to evaluate individual treatment effects at $P \leq 0.05$.

3. Results

3.1. High cadmium concentrations do not affect growth and development of soil-grown common ice plant

To determine the effect of Cd implementation on plant growth, plant biometric analyses were performed. None of the applied Cd concentrations induced visible morphological symptoms of heavy metal stress in either C_3- or CAM-performing plants. We found no significant differences in dry weights of root and shoot of control and cadmium-treated C_3- and CAM-performing ice plants (Table 2). Moreover, the shoot-to-root (DW) ratio demonstrated that both root and shoot growth were unaffected by the used Cd treatments (Table 3). Additionally, the analysis of the tissue water content (TWC), showed that none of the applied Cd concentrations had a detrimental effect on the water status of either root or shoot of C_3- and CAM-performing ice plants (supplementary data Fig. S1). Collectively, the biometric data confirmed a minute influence of the applied Cd concentrations on plant growth and water management.

Table 2
Dry weight of roots and shoots of soil-grown C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to the elevated concentrations of Cd.

Cadmium concentration [mM]	root DW [g/plant]		shoot DW [g/plant]	
	C_3	CAM	C_3	CAM
0	0.06 ± 0.011b	0.1 ± 0.003a	0.98 ± 0.22bc	1.47 ± 0.11a
0.01	0.09 ± 0.003ab	0.07 ± 0.009ab	1.07 ± 0.13abc	1.28 ± 0.25abc
0.1	0.07 ± 0.007ab	0.1 ± 0.012ab	0.85 ± 0.19c	1.3 ± 0.13ab
1	0.07 ± 0.013b	0.09 ± 0.008ab	0.91 ± 0.24bc	1.49 ± 0.2a

Means within columns followed by the same letters are not significantly different at $P < 0.05$ according to Tukey's test (N = 4, mean value ± SD).

Table 3
Shoot-to-root (DW) ratio in C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to increased Cd concentrations.

Cadmium concentration [mM]	shoot-to-root (DW) ratio	
	C_3	CAM
0	15.18 ± 1.37a	15.20 ± 3.39a
0.01	11.52 ± 1.8a	17.35 ± 2.2a
0.1	11.58 ± 1.98a	13.78 ± 2.19a
1	13.37 ± 2.07a	16.16 ± 0.92a

Means within columns followed by the same letters are not significantly different at $P < 0.05$ according to Tukey's test (N = 4, mean value ± SD).

3.2. Cadmium does not alter the efficiency of the photosynthetic apparatus

To assess the effect of increased Cd concentration on the performance of the photosynthetic apparatus, chlorophyll *a* fluorescence was analysed. The maximum quantum yield of PSII (Fv/Fm) of untreated CAM plants was significantly lower in comparison to the untreated C_3 plants (Table 4); this discrepancy was sustained under the lowest applied Cd concentration (0.01 mM). In C_3 plants, the Fv/Fm was not affected by any of the applied Cd concentrations and remained within the range from 0.82 to 0.83. In contrast to C_3 plants, the CAM plants revealed an increasing tendency of Fv/Fm accompanying Cd administration, and two of the applied Cd concentrations, precisely, 0.1 and 1 mM, caused a substantial increase when compared with the control. Photosynthetic performance index (PI) describes the overall photosynthetic performance of PSII. None of the administered Cd treatments had a detrimental effect on plants PI of both metabolic groups (Table 4). As in the case of Fv/Fm, two of the applied Cd concentrations, precisely 0.1 and 1 mM, caused a substantial increase of CAM plants' PI when compared with the control.

3.3. Roots of CAM-performing plants accumulated higher Cd amounts than C_3 as a result of higher Cd availability

To estimate the amount of cadmium available for the plant, we measured the bioavailable Cd level in the substrate after the plants were harvested. In substrates of untreated (control) C_3 and CAM plants low (near the detection limit) concentrations of bioavailable Cd were found (Fig. 1A). This result was later confirmed with detection of low Cd levels accumulated in the roots of intact C_3 and CAM plants, suggesting heavy metals contamination of the soil used for substrate preparation (see discussion). When the results were compared in a concentration-dependent manner, no substantial differences were found in the Cd bioavailability of plants' substrates treated with up to 0.1 mM. Moreover, bioavailable Cd concentration measured in these variants were low and remained in the range between 0.16–1.43 mg kg^{-1}. On the other hand, in CAM plants' substrates subjected the concentration of 1 mM we found the highest bioavailable Cd concentration, over 100-fold higher in comparison to that of C_3 plants. Amounts of Cd accumulated in the roots in most cases reflected the pattern of bioavailable

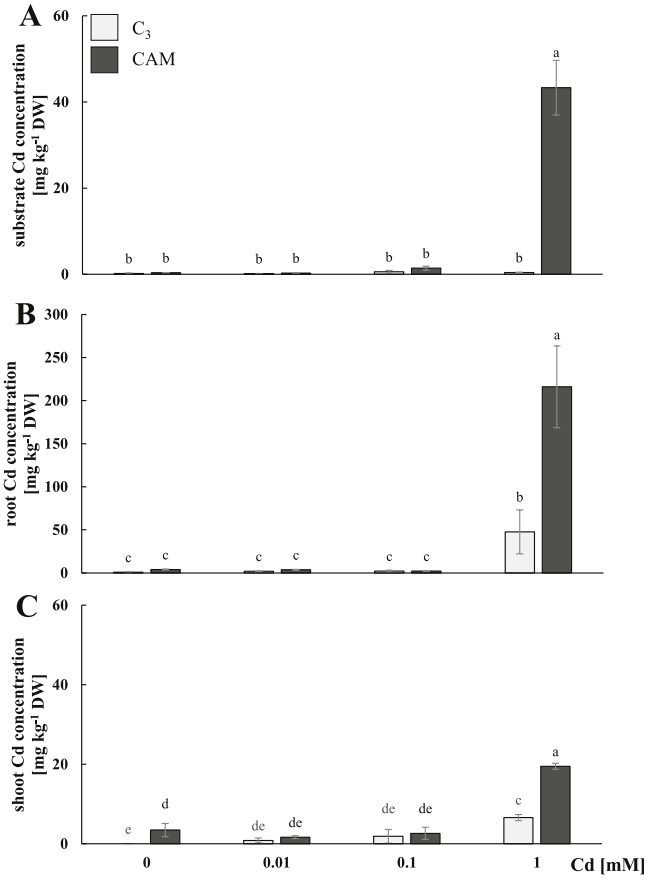

Fig. 1. Cadmium amounts measured in the substrate (A), roots (B) and shoots (C) of soil-grown C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to the concentration of 0 (control), 0.01, 0.1 and 1 mM of Cd. The substrate mass used per pot was 360 ± 0.1 g. Different letters above the bars indicate statistically significant differences at $P \leq 0.05$ by Tukey's post-hoc test (N = 4, mean value ± SD).

Cd concentrations measured in corresponding substrates, with the highest Cd accumulation, precisely 216.05 mg kg^{-1}, found in roots of CAM plants subjected to 1 mM Cd treatment (Fig. 1B). The exception was found in the roots of C_3 plants treated with the 1 mM Cd solution, accumulating over 47 mg kg^{-1} of Cd, what did not correlate with bioavailable Cd concentration measured in corresponding substrate sample (0.42 mg kg^{-1} Cd). Shoot-accumulated Cd resembled the pattern of Cd concentration observed in the roots (Fig. 1C), with the highest Cd amount, precisely 19.5 mg kg^{-1} of Cd, accumulated in CAM shoots under 1 mM treatment.

Table 4
Maximum quantum efficiency of PSII (Fv/Fm) and photosynthetic performance index (PI) of soil-grown C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to the increased Cd concentrations.

Cadmium concentration [mM]	Fv/Fm		PI	
	C_3	CAM	C_3	CAM
0	0.829 ± 0.014a	0.799 ± 0.007c	8.66 ± 1.35abcd	7.33 ± 1.06d
0.01	0.833 ± 0.011a	0.808 ± 0.005bc	7.97 ± 1.26bcd	7.91 ± 1.5cd
0.1	0.828 ± 0.007a	0.822 ± 0.01ab	8.49 ± 1.42abcd	9.95 ± 1.34abc
1	0.834 ± 0.01a	0.820 ± 0.009ab	10.81 ± 1.6a	10.29 ± 2.2ab

Means within columns followed by the same letters are not significantly different at $P < 0.05$ according to Tukey's test (N = 4, mean value ± SD).

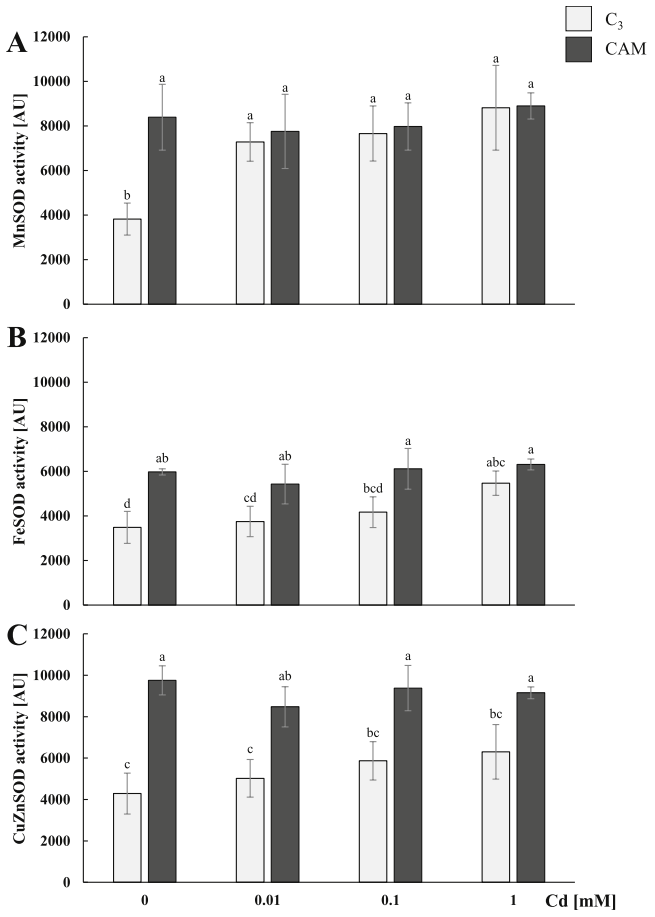

Fig. 2. The activity of manganese (A), iron (B) and copper/zinc (C) superoxide dismutase (SOD) of the soil-grown C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to the concentration of 0 (control), 0.01, 0.1 and 1 mM of Cd. The substrate mass used per pot was 360 ± 0.1 g. Different letters above the bars indicate statistically significant differences at $P \leq 0.05$ by Tukey's post-hoc test (N = 4, mean value ± SD).

3.4. CAM-performing plants represent a higher potential for Cd stress-derived superoxide radical detoxification. Cd induces SOD activity of C_3 plants in a compartment-dependent manner

To assess the effect of cadmium on the first-line of cell antioxidative defence, activities of three well-described SOD forms were determined (supplementary data Fig. S2). In our previous study, we confirmed that the activity of all the tested SOD forms found in intact C_3 plants was substantially lower than that in CAM plants (Nosek et al., 2015a). Here, we confirmed results of the mentioned study and showed that in the CAM metabolic group, the activity of all SOD forms was not affected by any of the applied cadmium concentrations (Fig. 2A, B, C). Moreover, we found that under all Cd treatments, the CuZnSOD activity of CAM plants remained significantly higher than that of C_3 plants (Fig. 2C), allowing more efficient scavenging of superoxide radical ($O_2^{\cdot -}$) generated in the cytoplasm.

In C_3 plants, the response of the MnSOD (mitochondrial) form was the most pronounced; after treatment with the lowest concentration (0.01 mM), the MnSOD activity significantly increased, reaching the level observed in CAM-performing plants. The response manner of MnSOD was similar for all the applied Cd concentrations (Fig. 2A). The activity of the FeSOD (chloroplastic) form of the C_3 plants was significantly induced to the level found in CAM plants, however, only by the concentration of 1 mM Cd (Fig. 2B). In the case of the CuZnSOD (cytoplasmic) form, no significant increase of activity was observed in C_3-plants response to elevated Cd concentrations (Fig. 2C).

Table 5
Rate of ^{13}C isotope discrimination in C_3- and CAM-performing *Mesembryanthemum crystallinum* plants subjected to increased Cd concentrations.

Cadmium concentration [mM]	$\delta^{13}C$	
	C_3	CAM
0	−29.4 ± 0.71b	−26.7 ± 1.10a
0.01	−30.4 ± 0.46b	−25.5 ± 0.94a
0.1	−30.5 ± 1.03b	−25.6 ± 0.04a
1	−29.8 ± 1.22b	−26.5 ± 0.97a

Means within columns followed by the same letters are not significantly different at $P < 0.05$ according to Tukey's test (N = 4, mean value ± SD).

3.5. Cadmium stress does not induce the shift from C_3 to CAM

As previously shown for *M. crystallinum,* the plants respond to some abiotic stresses by initiating a shift from C_3 to CAM (Winter and Holtum, 2014). In this study, the Δ malate concentration (the difference between malate concentration measured at the beginning and the end of the light phase), a hallmark of CAM, was within the ranges 2.7–4.6 and 18.2–23.8 mM in C_3- and CAM-performing common ice plants subjected to Cd concentrations, respectively (data not shown). For additional confirmation of *β*-carboxylation occurrence, a ^{13}C isotope discrimination was employed based on the determination of ^{13}C isotope amount fixed within plant tissues (Table 5). Distinctive alterations in the level of ^{13}C discrimination were observed only between C_3- and CAM-performing plants, similar to those described in our previous study (Nosek et al., 2015b). None of the applied Cd treatments affected ^{13}C discrimination of C_3-performing plants. Both these analyses confirmed lack of CAM induction in C_3 plants under Cd treatment.

4. Discussion

While cadmium detrimental effects on plant growth and development have been confirmed, no important role in plant physiology has been described yet (Rascio and Navari-Izzo, 2011). The only reports suggesting its participation in the physiological processes of photoautotrophs comes from marine diatom - *Thalassiosira weissglogii* - where Cd was found in the active metal-binding site of carbonic anhydrase (Lane and Morel, 2000; Lane et al., 2005). Certain quantities of Cd present in the rhizosphere can easily enter a plant, exploiting divalent transporters, *e.g.*, IRT (iron-regulated transporter), which is a part of the ZIP (**Z**RT, **I**RT-like **P**rotein) family, dedicated for the uptake of essential nutrients (Vert et al., 2001, 2002). According to previous studies regarding the interaction between Cd and hydroponically cultured common ice plant, the plant biomass yield was significantly affected with Cd concentration of 10 μM, and this effect tended to intensify with concentration increase (Shevyakova et al., 2003). Additionally, the exposure to 50 μM Cd induced a dramatic decrease in chlorophyll concentration and dry weight of perlite-grown common ice plant (Ghnaya et al., 2005). In our experiments, the soil-grown *M. crystallinum* plants of two metabolic types, namely, C_3 and CAM, were treated with elevated Cd concentrations. To date, none of the studies concerning the ice plant-Cd interaction have considered a potential role of photosynthetic metabolism during such response (Thomas et al., 1998; Kholodova et al., 2005; Amari et al., 2014). In our study, biometric analyses confirmed no visible disturbances in the growth and development of ice plants treated with Cd concentrations up to 1 mM. Both dry weight and the shoot-to-root ratio of C_3- and CAM-performing plants were not affected by any of the Cd treatments. Lack of detrimental effects of Cd was also confirmed with the undisturbed efficiency of the photosynthetic apparatus found in cadmium-treated plants; both the maximum quantum efficiency of PSII (Fv/Fm) and photosynthetic performance index (PI) remained unaltered in Cd-treated plants. Earlier

studies performed on isolated PSII-enriched membranes (spinach) and *Chlamydomonas reinhardtii* showed that in the low μM ranges Cd disrupts the assembly of water splitting complex due to competition at Ca^{2+} site of PSII (Faller et al., 2005). Water deficit has long been identified as one of the earliest manifestations of HMs toxicity (Lefèvre et al., 2009). In hydroponically-grown ice plants subjected to increased doses of Cu and Zn the loss of relative and tissue water content (TWC), as well as the accumulation of proline, the major osmoprotective aminoacid, was observed (Kholodova et al., 2011). Moreover, Ghnaya et al. (2005) established that hydroponically grown ice plants subjected to 300 μM Cd decreased their leaf water content by 40%. In our study, we found no evidence of the detrimental effect of the applied Cd concentrations on the water status of either C_3 or CAM-performing common ice plants. Altogether these results suggest that despite the application of elevated Cd quantities to the substratum, the metal either remained unavailable for plants or as available was sequestered and deposited in dedicated organs/tissues to secure the main metabolic pathways from its harmful effects.

To determine the amount of heavy metal that interacted with the plant, we measured bioavailable Cd content in the substrate of both treated and untreated plants. Our analysis unequivocally confirmed Cd contamination of the soil substrate used in the study. As a result, low heavy metal concentrations were detected in roots of control (untreated) C_3 and CAM plants. It was not surprising since most of the market available soils are supplied with fertilizers, which are widely recognized as the primary source of heavy metal (primarily Cd and Pb) contamination. Moreover, analysis of bioavailable Cd concentration suggests that up to 0.1 mM most of substrate-introduced Cd was not available for plant uptake, probably remaining immobilized in a solid matrix. Soil pH, anionic adsorption and organic matter content are considered as the decisive factors affecting the biological availability of heavy metals (Prasad, 2004). Recently, also the effect of salts, mainly chlorides, on heavy metals bioavailability and uptake has been pronounced (Lutts and Lefèvre, 2015). In wheat, corn, and duckweed the introduction of chloride salts into soil substrate, primarily NaCl, increased the amount of soluble Cd and therefore its uptake (Weggler et al., 2004; Ghallab and Usman, 2007; Du Laing et al., 2008). In our experimental setup, the sodium chloride pre-treatment was utilized for CAM induction in *M. crystallinum*. However, the visible effect of salt-enhanced Cd bioavailability was observed only in substrate subjected to the highest Cd concentration, namely 1 mM. It suggests that under lower Cd treatments salt-derived ions could not successfully overcome immobilizing potential of the solid matrix. In the case of C_3 plants, low ($0.42\ mg\ kg^{-1}$) concentration of bioavailable Cd measured in the substrate treated with 1 mM was accompanied by elevated Cd accumulation in the corresponding root and shoot samples. These suggest that under 1 mM treatment C_3 plants had access to elevated, amounts of bioavailable Cd and uptaken most of it from the substrates.

To elucidate how substrate Cd bioavailability affected the heavy metal accumulation in plant tissues, we analysed cadmium concentrations in the roots and shoots of C_3- and CAM-performing ice plants. Shevyakova et al. (2003) established that *M. crystallinum* plants exposed to a micromolar range of Cd concentrations accumulated amounts typical of hyperaccumulators; however, the process occurred primarily in the root apoplast with only small translocation towards the aerial parts. Our experiments confirmed this result for both metabolic states tested. Thus, we concluded that neither C_3- nor CAM-performing ice plants could be considered as Cd hyperaccumulators, although they had some features of hyperaccumulators. For example, common ice plants demonstrated increased resistance to excessive Cd concentration and without visible morphological symptoms, executed the vegetative part of the developmental programme by accumulating small amounts of the heavy metal also in the shoots. According to van der Ent et al. (2013), in addition to the well-recognized strategies aimed at coping with HM stress shown by insensitive (accumulators and hyperaccumulators) and sensitive representatives, so-called excluders to occur. These plants are able to restrict heavy metal uptake until the concentration in the substratum exceeds a specific threshold. The results of this work suggested that the soil-grown *M. crystallinum* employed this strategy; however, C_3- and CAM-performing plants pursued the strategy in different ways. Our results showed that at the highest applied concentration (1 mM), C_3 plants had access to significantly lower amounts of bioavailable Cd in comparison to CAM plants, where the NaCl pre-treatment significantly extended heavy metal bioavailability. As a result, roots of CAM plants accumulated over 4-fold higher amount of Cd in comparison to that of C_3 plants. Summarizing, significant differences in the rate of Cd accumulation found between plants of both metabolic groups resulted from NaCl pre-treatment, rather than the performance of CAM photosynthesis. In the halophytic species *Atriplex halimus*, NaCl treatment led to decreased levels of accumulated Cd in comparison with untreated plants. Based on this finding, a mechanism of improved resistance to Cd toxicity was proposed, which relied on reduced heavy metal absorption and increased osmoprotectant synthesis in salt-treated plants (Lefèvre et al., 2009). Contrary to this study, our experiments showed that NaCl pre-treatment enhanced Cd-accumulating potential of *M. crystallinum* plants, confirming that osmotic stress episode made ice plants well-prepared for the occurrence of the additional stressor, in this particular case, heavy metal. CAM induction in *M. crystallinum* is accompanied by mobilization of antioxidative system components, modified reactive oxygen species (ROS) generation and reorganization of photosystems; collectively, these changes result in extended resistance to different abiotic and biotic stresses (Niewiadomska and Miszalski, 2008; Ślesak et al., 2008; Libik-Konieczny et al., 2011; Niewiadomska et al., 2011; Gabara et al., 2012). As previously stated, increased Cd concentration initiates ROS overproduction responsible for oxidative stress (Schützendübel and Polle, 2002). We believe that enhanced accumulating potential for Cd confirmed in CAM-performing plants was possible due to extended antioxidative protection of NaCl pre-treated plants. To test this hypothesis, we analysed the superoxide dismutase (SOD) activity in plants of both metabolic groups subjected to Cd stress. Here, we confirmed our previous findings characterizing CAM-performing plants with increased activity of SOD (Miszalski et al., 1998; Nosek et al., 2015a, 2018). Our results suggested that SOD activity once established in CAM plants during salinity stress was sufficiently high to enable removal of additional quantities of $O_2^{\cdot -}$ generated in the response to Cd. Increased antioxidative potential of NaCl pre-treated plants was pronounced with significantly higher activity of CuZnSOD, not achievable by C_3 plants despite high Cd concentrations applied. Such induction of SOD activity allowed efficient removal of overproduced $O_2^{\cdot -}$ and thus, may justify the enhanced Cd-accumulating potential of CAM plants. By contrast, in C_3 plants, the lowest applied Cd concentration initiated intensified $O_2^{\cdot -}$ generation in mitochondria that was reflected in induced MnSOD activity. Small alterations of FeSOD and CuZnSOD activities confirmed the compartment-dependent induction of $O_2^{\cdot -}$ overproduction in response to applied Cd concentrations. Although the alterations of SOD activity found in C_3 plants suggested excessive ROS generation as a result of elevated amounts of Cd in some cell compartments, the redox balance response was finely tuned probably by other the antioxidative system components, and therefore, no visible symptoms of oxidative damage occurred.

Common ice plant is a well-described CAM facultative species, known for an inducible and reversible shift from C_3 to CAM (Cushman and Bohnert, 1997; Adams, 1998; Lüttge, 2004; Winter and Holtum, 2014; Nosek et al., 2018). In an earlier study, when treated with micromolar concentrations of $CuSO_4$ and $ZnSO_4$, hydroponically grown common ice plants shifted metabolism from C_3 to CAM within 7 days (Kholodova et al., 2011). Although excessive concentrations of heavy metals are categorized as abiotic-type stressors, we confirmed in this study that the applied Cd concentrations did not initiate the photosynthetic metabolism shift noticeable in terms of the Δ malate changes or modified ^{13}C discrimination.

5. Conclusion

In this study, we confirmed high resistance of both metabolic types (C_3 and CAM) of a soil-grown halophyte, the common ice plant, to increased concentrations of Cd. We propose that *M. crystallinum* in presence of high Cd concentrations can utilize an excluding strategy. However, this strategy was implemented differently by C_3- and CAM-performing plants and the enhanced accumulating potential observed in CAM plants was rather a result of osmotic stress episode than β-carboxylation pathway occurrence. We confirmed that extended resistance of CAM plants towards Cd toxicity was possible also due to the enhanced antioxidative capacity. Cd treatment did not induce CAM. To summarize, the common ice plants represent features useful in phytoremediation of high salinity areas simultaneously polluted with Cd and so reveal the high potential in terms of environmental biotechnology applications.

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Appendix A. Supplementary data

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References

Adams, P., Nelson, D.E., Yamada, S., Chmara, W., Jensen, R.G., Bohnert, H.J., Griffiths, H., 1998. Growth and development of *Mesembryanthemum crystallinum (Aizoaceae)*. New Phytol. 138, 171–190.

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals – concepts and application. Chemosphere 91, 869–881.

Amari, T., Ghnaya, T., Debez, A., Taamali, M., Youssef, N., Lucchini, G., Sacchi, G.A., Abdelly, C., 2014. Comparative Ni tolerance and accumulation potentials between *Mesembryanthemum crystallinum* (halophyte) and *Brassica juncea*: metal accumulation, nutrient status and photosynthetic activity. J. Plant Physiol. 171, 1634–1644.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in plants. Braz. J. Plant Physiol. 17, 21–34.

Borland, A., Elliot, S., Patterson, S., Taybi, T., Cushman, J., Pater, B., Barnes, J., 2006. Are the metabolic components of crassulacean acid metabolism up-regulated in response to an increase in oxidative burden? J. Exp. Bot. 57, 319–328.

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

Cushman, J.C., Michalowski, C., Bohnert, H.J., 1990. Developmental control of Crassulacean acid metabolism inducibility by salt stress in the common ice plant. Plant Physiol. 94, 1137–1142.

Cushman, J.C., Bohnert, H.J., 1997. Molecular genetics of Crassulacean acid metabolism. Plant Physiol. 113, 667–676.

Das, P., Samantaray, S., Rout, G.R., 1997. Studies on cadmium toxicity in plants. A review. Environ. Pollut. 98, 29–36.

Du Laing, G., De Vos, R., Vandecasteele, B., Lesage, E., Tack, F.M.G., Verloo, M.G., 2008. Effect of salinity on heavy metal mobility and availability in intertidal sediments of the Scheldt estuary. Estuar. Coast. Shelf Sci. 77, 589–602.

Faller, P., Kienzler, K., Krieger-Liszkay, A., 2005. Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of Photosystem II by competitive binding to the essential Ca^{2+} site. Biochim. Biophys. Acta 1706, 158–164.

Gabara, B., Kuźniak, E., Skłodowska, M., Surówka, E., Miszalski, Z., 2012. Ultrastructural and metabolic modifications at the plant-pathogen interface in *Mesembryanthemum crystallinum* leaves infected by *Botrytis cinerea*. Environ. Exp. Bot. 77, 33–43.

Gawronska, K., Niewiadomska, E., 2015. Participation of citric acid and isocitric acid in the diurnal cycle of carboxylation and decarboxylation in the common ice plant. Acta Physiol. Plant. 37, 61.

Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) – Gen. Subj. 990, 87–92.

Ghallab, A., Usman, A.R., 2007. Effect of sodium choride-induced salinity of phyto-availability and speciation of Cd in soil solution. Water Air Soil Pollut. 185, 43–51.

Ghnaya, T., Nouairib, I., Slamaa, I., Messedia, D., Grignonc, C., Abdellya, C., Ghorbel, M., 2005. Cadmium effects on growth and mineral nutrition of two halophytes: *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum*. J. Plant Physiol. 162, 1133–1140.

Halliwell, B., Gutterigde, J.M., 2015. Free Radicals in Biology and Medicine, 5th ed. .

Heyno, E., Klose, C., Krieger-Liszkay, A., 2008. Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. New Phytol. 179, 687–699.

Hurst, A.C., Grams, T.E.E., Ratajczak, R., 2004. Effects of salinity, high irradiance, ozone, and ethylene on mode of photosynthesis, oxidative stress and oxidative damage in the C_3/CAM intermediate plant *Mesembryanthemum crystallinum* L. Plant Cell Environ. 27, 187–197.

Kholodova, V., Volkov, K., Kuznetsov, V., 2005. Adaptation of the common ice plant to high copper and zinc concentrations and their potential using for phytoremediation. Russ. J. Plant Physiol.+ 52, 748–757.

Kholodova, V., Volkov, K., Abdeyeva, A., Kuznetsov, V., 2011. Water status in *Mesembryanthemum crystallinum* under heavy metal stress. Environ. Exp. Bot. 71, 382–389.

Kuznetsov, V., Neto, D.S., Borisova, N., Dam, Z.B., Yu, Rakitin V., Alexandrova, S.N., Kholodova, V., 2000. Stress-induced CAM development and the limit of adaptation potential in *Mesembryanthemum crystallinum* plants under extreme conditions. Russ. J. Plant Physiol.+ 47, 168–175.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lane, T.W., Morel, F.M.M., 2000. A biological function for cadmium in marine diatoms. Proc. Natl. Acad. Sci. U. S. A. 97, 4627–4631.

Lane, T.W., et al., 2005. A cadmium enzyme for a marine diatom. Nature 435, 42.

Lefèvre, I., Marchal, G., Meerts, P., Corréal, E., Lutts, S., 2009. Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ. Exp. Bot. 65, 142–152.

Libik-Konieczny, M., Surówka, E., Kuźniak, E., Nosek, M., Miszalski, Z., 2011. Effects of *Botrytis cinerea* and *Pseudomonas syringae* infection on the antioxidant profile of *Mesembryanthemum crystallinum* C_3/CAM intermediate plant. J. Plant Physiol. 168, 1052–1059.

Libik-Konieczny, M., Surówka, E., Nosek, M., Goraj, S., Miszalski, Z., 2012. Pathogen-induced changes in malate content and NADP-dependent malic enzyme activity in C_3 and CAM performing *Mesembryanthemum crystallinum* L. plants. Acta Physiol. Plant. 34, 1471–1477.

Lüttge, U., 2004. Ecophysiology of Crassulacean acid metabolism (CAM). Ann. Bot. 93, 629–652.

Lutts, S., Lefèvre, I., 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann. Bot.-Lond. 115, 509–528.

Maleki, M., Ghorbanpour, M., Kariman, K., 2017. Physiological and antioxidative responses of medicinal plants exposed to heavy metals stress. Plant Gene 11, 247–254.

Manousaki, E., Kalogerakis, N., 2011. Halophytes – an emerging trend in phytoremediation. Int. J. Phytoremediat. 13, 959–969.

Miszalski, Z., Ślesak, I., Niewiadomska, E., Baczek-Kwinta, R., Lüttge, U., Ratajczak, R., 1998. Subcellular localization and stress responses of superoxide dismutase isoforms from leaves in the C_3→CAM intermediate halophyte *Mesembryanthemum crystallinum* L. Plant Cell Environ. 21, 169–179.

Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M., 2010. Heavy metals, occurrence and toxicity for plants: a review. Environ. Chem. Lett. 8 (3), 199–216.

Niewiadomska, E., Miszalski, Z., 2008. Partial characterization and expression of leaf catalase in the CAM-inducible halophyte *Mesembryanthemum crystallinum* L. Plant Physiol. Biochem. 46, 421–427.

Niewiadomska, E., Bilger, W., Gruca, M., Mulisch, M., Miszalski, Z., Krupiska, K., 2011. CAM-related changes in chloroplastic metabolism of *Mesembryanthemum crystallinum* L. Planta 233, 275–285.

Nosek, M., Kornaś, A., Kuźniak, E., Miszalski, Z., 2015a. Plastoquinone redox state modifies plant response to pathogen. Plant Physiol. Biochem. 96, 163–170.

Nosek, M., Rozpądek, P., Kornaś, A., Kuźniak, E., Schmitt, A., Miszalski, Z., 2015b. Veinal-mesophyll interaction under biotic stress. J. Plant Physiol. 185, 52–56.

Nosek, M., Gawrońska, K., Rozpądek, P., Szechyńska-Hebda, M., Kornaś, A., Miszalski, Z., 2018. Withdrawal from functional *Crassulacean acid metabolism* is accompanied by changes in both genes expression and activity of antioxidative enzymes. J. Plant Physiol. 229, 151–157.

Ostrowska, A., Gawliński, S., Szczubiałka, Z., 1991. Metody analizy i oceny właściwości Gleb i roślin [Soil and Plant Analysis Procedures]. Wyd. Instytutu Ochrony Środowiska, Warsaw.

Pagliano, C., Raviolo, M., Dalla Vecchia, F., et al., 2006. Evidence for PSII donor-side damage and photoinhibition induced by cadmium treatment on rice (*Oryza sativa* L.). J. Photochem. Photobiol. B 84, 70–78.

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., et al., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ. Pollut. 215, 203–212.

Prasad, M.V.N., 2004. Heavy Metal Stress in Plants. From Biomolecules to Ecosystems, 2nd edition. ©Springer-Verlag, Heidelberg.

Rascio, N., Navari-Izzo, F., 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? Plant Sci. 180, 169–181.

Rozpądek, R., Domka, A., Ważny, R., Nosek, M., Jędrzejczyk, R., Tokarz, K., Turnau, K., 2018. How does the endophytic fungus *Mucor* sp. Improve *Arabidopsis arenosa* vegetation in the degraded environment of a mine dump? Environ. Exp. Bot. 147, 31–42.

Ślesak, I., Libik, M., Miszalski, Z., 2008. The foliar concentration of hydrogen peroxide during salt-induced C_3→CAM transition in *Mesembryanthemum crystallinum* L. Acta Biochim. Pol. 174, 221–226.

Schützendübel, A., Polle, A., 2002. Plant response to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351–1365.

Shaw, B.P., Sahu, S.K., Mishra, R.K., 2004. Heavy Metal Induced Oxidative Damage in Terrestrial Plants. Heavy Metal Stress in Plants. Springer-Verlag, Berlin, Heidelberg, pp. 84–126.

Shevyakova, N., Netronina, I., Aronova, E., Kuznetsov, V., 2003. Compartmentation of

cadmium and iron in *Mesembryanthemum crystallinum* plants during the adaptation to cadmium stress. Russ. J. Plant Physiol. + 50, 678–685.
Shukla, U.C., Murthy, R.C., Kakkar, P., 2008. Combined effect of ultraviolet-B radiation and cadmium contamination on nutrient uptake and photosynthetic pigments in *Brassica campestris* L. seedlings. Environ. Toxicol. 23, 712–719.
Strasser, R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus, M., Pathre, U., Mohanty, P. (Eds.), Probing Photosynthesis. CRC Press, London, pp. 445–483.
Thomas, J.C., Malick, F.K., Endreszl, C., Davies, E.C., Murray, K., 1998. Distinct responses to copper stress in the halophyte *Mesembryanthemum crystallinum*. Physiol. Plant. 102, 360–368.
Vert, G., Briat, J.F., Curie, C., 2001. Arabidopsis IRT2 gene encodes a root-periphery iron transporter. Plant J. 26, 181–189.
Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J.F., Curie, C., 2002. IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. Plant Cell 14, 1223–1233.
Weggler, K., McLaughlin, M.J., Graham, R.D., 2004. Effect of chloride in soil solution on the plant availability of biosolid-borne cadmium. J. Environ. Qual. 33, 496–504.
Winter, K., Garcia, M., Holtum, J.A.M., 2008. On the nature of facultative and constitutive CAM: environmental and developmental control of CAM expression during early growth of Clusia, Kalanchoë, and Opuntia. J. Exp. Bot. 59, 1829–1840.
Winter, K., Holtum, J.A.M., 2014. Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis. J. Exp. Bot. 65, 3425–3441.
Van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J., Schat, H., 2013. Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant Soil 362, 319–334.