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Characterization of Arabidopsis species from metalliferous and non-metalliferous sites in Southern Poland

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1. STRESZCZENIE

Zanieczyszczenie środowiska metalami ciężkimi jest wciąż wysokie, jako efekt naturalnych procesów oraz działalności człowieka. Obecność metali toksycznych, takich jak Cd lub bardzo wysoka zawartość mikroelementów, takich jak Zn, stanowią poważne zagrożenie dla środowiska. Rośliny pobierają metale z gleby i akumulują je w swoich tkankach, co może stanowić zagrożenie dla zdrowia ludzi spożywających rośliny, zawierające wysokie stężenia tych metali. Dlatego istnieje potrzeba opracowania nowych i skutecznych technik usuwania metali ciężkich ze środowiska lub ograniczania ich negatywnego wpływu na organizmy żywe.

Celem projektu było pogłębienie wiedzy na temat mechanizmów związanych z pobieraniem i akumulacją metali przez rośliny oraz odpornością na ich toksyczne działanie. Hyperakumulatory metali ciężkich to rośliny, które mogą żyć na glebach silnie zanieczyszczonych przez metale i posiadają zdolność do akumulacji niezwykle wysokich stężeń tych metali w tkankach nadziemnych. *Arabidopsis halleri* wykorzystywane jest jako gatunek modelowy do badania mechanizmów związanych z tolerancją i akumulacją wysokich stężeń metali. Gatunek ten charakteryzuje się wysoką odpornością na duże stężenia Cd i Zn i jest zdolny do hyperakumulacji Zn w pędach, a także Cd w przypadku niektórych populacji. *Arabidopsis arenosa*, gatunek blisko spokrewniony z *A. halleri*, również wykazuje wysoką odporność na oba metale. Wcześniejsze badania wykazały, że *A. arenosa* akumuluje metale głównie w korzeniach, transportując jedynie niewielką część Cd i Zn z korzeni do pędów. W południowej Polsce oba gatunki można zaobserwować na tych samych stanowiskach silnie zanieczyszczonych metalami. Eksperymenty miały na celu zbadanie między- i wewnątrzgatunkowej zmienności pomiędzy populacjami *A. halleri* i *A. arenosa* z terenów czystych oraz zanieczyszczonych metalami, w celu zidentyfikowania parametrów fizjologicznych i genów odpowiedzialnych za różnice w ich adaptacji do toksycznych stężeń metali. Aby odpowiedzieć na postawione pytania badawcze, pracę podzielono na trzy etapy, stanowiące odrębne rozdziały.

Pierwszy rozdział obejmuje badania terenowe oraz eksperymenty laboratoryjne. W celu określenia różnic fizjologicznych w ich naturalnym środowisku oraz przy traktowaniu różnymi stężeniami Cd w hydroponice, przebadano po jednej populacje z terenów czystych oraz po jednej z trenów zanieczyszczonych metalami, zarówno dla *A. halleri* jak i *A. arenosa*. Przeanalizowano całkowitą zwartość metali w glebie oraz biodostępne frakcje Cd, Pb i Zn.

Zawartość tych pierwiastków oznaczono również w korzeniach i liściach wszystkich badanych populacji. Ponadto porównano aktywność aparatu fotosyntetycznego i zawartość barwników we wszystkich czterech populacjach rosnących w ich naturalnym środowisku. Ploidalność każdej populacji została przeanalizowana w celu potwierdzenia, że badane populacje *A. halleri* są diploidalne, natomiast *A. arenosa* tetraploidalne. Dzięki temu możliwe było wykluczenie wymiany materiału genetycznego między dwoma gatunkami rosnącymi na tym samym stanowisku. W warunkach hydroponicznych rośliny poddano działaniu 50, 100 i 200 μM Cd przez 24 dni. Następnie przeprowadzono taką samą analizę jak *in situ*. Po raz pierwszy przeanalizowano skład mineralny *A. arenosa* w połączeniu z analizą aktywności aparatu fotosyntetycznego zarówno w warunkach terenowych, jak i laboratoryjnych. Moje wyniki pokazują, że populacja *A. arenosa* z terenu zanieczyszczonego metalami jest tak samo tolerancyjna na metale ciężkie, jak hyperakumulująca populacja *A. halleri* z tego samego stanowiska. Ponadto moje wyniki wykazały, że populacja *A. arenosa* z terenu zanieczyszczonego metalami hyperakumuluje Cd i Zn.

Drugi rozdział przedstawia analizę odpowiedzi fizjologicznej populacji *A. halleri* i *A. arenosa* ze stanowiska zanieczyszczonego metalami, traktowanych wysokim stężeniem Cd (1.0 mM) lub Zn (5.0 mM). Różnice w akumulacji Cd i Zn, tolerancji, aktywności aparatu fotosyntetycznego i zmiany zawartości barwników zostały przeanalizowane po 7 dniach traktowania. Populacja *A. arenosa* akumulowała więcej Cd w porównaniu z populacją *A. halleri*, która akumulowała więcej Zn. Traktowanie wysokim stężeniem Cd i Zn potwierdziło bardzo wysoki poziom tolerancji populacji *A. arenosa* i *A. halleri* z terenu zanieczyszczonego na oba metale. Oba gatunki różniły się zmianami w aktywności aparatu fotosyntetycznego w odpowiedzi na Cd i Zn w wysokich stężeniach, co sugeruje różnice w mechanizmach homeostazy metali pomiędzy gatunkami.

Trzeci rozdział skupia się na ocenie korelacji pomiędzy odpowiedzią fizjologiczną i ekspresją genów w populacjach *A. halleri* i *A. arenosa* z terenów czystych, jak i zanieczyszczonych metalami. Rośliny traktowano 5 μM Cd lub 150 μM Zn przez 10 dni w kulturach hydroponicznych. Do analizy ekspresji genów w warunkach umiarkowanego stresu zarówno w hypertolerancyjnych, jak i wrażliwych populacjach wybrano niższe stężenia metali, niż w pierwszym eksperymencie. W trakcie badań określono wzrost roślin, poziom tolerancji na Cd i Zn, skład mineralny korzeni i liści oraz zawartość barwników w liściach. Ponadto zmierzono poziom ekspresji genów zaangażowanych w pobieranie, translokację lub

detoksykację Cd i Zn oraz biosyntezę flawonoidów (*HMA2*, *HMA3*, *HMA4*, *MTP1*, *IRT1*, *IRT3*, *FRD3*, *FRO2*, *ZIF1*, *NRAMP3*, *NRAMP4*, *OPT3*, *NAS2*, *LDOX* i *F3H*). Zaobserwowano wyraźną różnicę w akumulacji Cd i Zn pomiędzy populacją *A. halleri* i *A. arenosa* z terenów zanieczyszczonych. Analiza poziomu ekspresji genów wykazała różnice nie tylko między gatunkami, ale także między populacjami. Traktowanie Cd lub Zn miało również różny wpływ na wszystkie populacje w odniesieniu do niektórych genów.

Wyniki uzyskane w ramach tego projektu wypełniają lukę w charakterystyce fizjologicznej gatunku *A. halleri* zarówno w warunkach terenowych, jak i w kulturach hydroponicznych. Co więcej, dogłębna charakterystyka fizjologiczna populacji *A. arenosa* z terenu zanieczyszczonego pozwoliła na zidentyfikowanego go, jako nowego hyperakumulatora Cd i Zn. Hyperakumulująca populacja *A. arenosa* z terenów metalonośnych może zostać wykorzystana, jako nowy model do przyszłych badań nad zjawiskiem hyperakumulacji. Co więcej, zaobserwowane różnice w ekspresji genów zaangażowanych w pobieranie, translokację i detoksykację metali między *A. halleri* i *A. arenosa* sugeruje, że mechanizmy homeostazy metali u *A. arenosa* są różne od *A. halleri*, i dalsze badania z wykorzystaniem obu gatunków przyczynią się do lepszego zrozumienia tych mechanizmów.

2. SUMMARY

Environmental pollution with trace metal elements (TMEs) has been high due to natural and anthropogenic sources. The presence of toxic metals such as Cd or very high content of essential micronutrients such as Zn poses a serious threat to the environment. Plants take up TMEs from the soil and accumulate them in their tissues, which can pose a threat for health of humans consuming plants containing higher concentrations of these metals. Therefore, there is a need to develop new and effective techniques of removal of heavy metals form the environment or limiting their negative influence on living organisms.

The aim of the project was to acquire deeper knowledge on the mechanisms involved in the uptake and accumulation of trace metal elements (TME) by plants and in the resistance to their toxic effects. The so-called metal hyperaccumulators, as *Arabidopsis halleri*, are plants that can live on soils heavily polluted by TME and have the ability to accumulate extraordinarily high concentrations of these metals in above-ground tissues. *A. halleri* can be used as a model-species to study the biological and molecular mechanisms involved in the tolerance and accumulation of high concentration of metals in the soil. This species is characterized by high tolerance to high concentrations of Cd and Zn. It is also capable of accumulating very large quantities of Zn in the shoots, named Zn hyperaccumulators, and also of Cd in some populations, named Cd hyperaccumulators. *Arabidopsis arenosa*, a closely related species to *A. halleri*, also shows high tolerance to both metals but is reported as a non-hyperaccumulator. Previous studies showed that *A. arenosa* accumulates metals mainly in the roots, translocating only a small portion of Cd and Zn from roots to shoots. In Southern Poland both species can be observed on the same sites. The experiments were designed to study inter and intra –specific variability between metallicolous and non- metallicolous populations of *A. halleri* and *A. arenosa* in order to identify physiological parameters and genes responsible for contrasting behaviour in their adaptation to metal contamination. In order to achieve the goal, the investigations were divided into stages that constitute separate chapters in this work.

First chapter includes field investigations as well as laboratory experiments. Both one metallicolous and one non-metallicolous population of *A. halleri* and *A. arenosa* (four populations in total) from Southern Poland were thoroughly investigated to determine physiological differences in their native site and upon different Cd treatments in the hydroponic experiments. The total concentration of TMEs and bioavailable fractions of Cd, Pb and Zn in the original soil were analysed. The concentration of these elements was also analysed in roots

and leaves of all investigated populations. Moreover, the photosynthetic apparatus activity and pigment content were compared in all populations growing in their natural habitat. The ploidy of each population was analysed to confirm that investigated populations of *A. halleri* are diploid and *A. arenosa* are tetraploid to rule out genetic material exchange between two species. In hydroponic culture plants were exposed to 50, 100 and 200 μM of Cd for 24 days. Afterwards the same analyses as *in situ* were performed. For the first time mineral composition of *A. arenosa* combined with analysis of photosynthetic apparatus activity was performed both in field and in controlled conditions. Our results show that metallicolous *A. arenosa* is as tolerant to heavy metals as the metallicolous population of hyperaccumulator *A. halleri*. Moreover, the metallicolous population of *A. arenosa* was shown to hyperaccumulate Cd and Zn.

The second chapter contains the analysis of the response of metallicolous populations of *A. halleri* and *A. arenosa* exposed to extremely high Cd (1.0 mM) or Zn (5.0 mM) concentration. Differences in Cd and Zn accumulation, tolerance, photosynthetic apparatus performance and pigment content changes were assessed after 7 days of treatment. *A. arenosa* accumulated more Cd than *A. halleri* while the latter accumulated more Zn. Treatment with extreme Cd and Zn concentrations confirmed extreme level of tolerance of both metallicolous *A. arenosa* and *A. halleri* populations. Both species differed in the response of photosynthetic apparatus when exposed to Cd and Zn at high concentration, suggesting different mechanisms involved in metal homeostasis.

The third chapter focused on evaluation of the correlation between physiological responses and gene expression in *A. halleri* and *A. arenosa* metallicolous and non metallicolous populations. In this chapter expression of genes underlying physiological differences between the metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa* was analysed. Plants were treated in hydroponic experiment with 5 μM Cd or 150 μM Zn for 10 days. Lower concentration of metals than in 1st experiment was chosen to analyse the gene expression under mild stress in both hypertolerant metallicolous and sensitive non-metallicolous populations. During the treatment growth of plants, level of Cd and Zn tolerance, mineral composition and pigment content was analysed. The expression level of genes involved in the uptake, translocation or detoxification of Cd and Zn, as well as the biosynthesis of flavonoids (*HMA2*, *HMA3*, *HMA4*, *MTP1*, *IRT1*, *IRT3*, *FRD3*, *FRO2*, *ZIF1*, *NRAMP3*, *NRAMP4*, *OPT3*, *NAS2*, *LDOX* and *F3H*) was analysed. We observed contrasting accumulation of Cd and Zn in *A. halleri* and *A. arenosa* metallicolous populations. Expression level of genes showed differences

not only between species but also between populations. The type of treatment (Cd or Zn) also had different effect on all populations in regards to some genes.

The results acquired in this project fill the gap in the physiological characteristics of *A. halleri* species both in the field and in controlled hydroponic conditions. The in depth physiological characterisation of *A. arenosa* from metalliferous site identified this population as a new hyperaccumulator of Cd and Zn. These results may serve as a good basis for future studies on the phenomenon of hyperaccumulation. Moreover, observed the contrasting expression of genes involved in metal uptake, translocation and detoxification between *A. halleri* and *A. arenosa* suggests that *A. arenosa* might be a good new model to study metal homeostasis and tolerance in plants.

3. INTRODUCTION

3.1. Trace metal elements in the environment

Trace metal elements (TME) such as cadmium and zinc are commonly found in soils worldwide. Natural sources of TME consist of e.g. weathering and erosion of parent rock, volcanic emissions, atmospheric deposition, evaporation of oceans, forest fires and decomposition of organic material (Kabata-Pendias & Szteke, 2015; Małkowski et al., 2019). However, since industrialization, anthropogenic sources became the major factor in deposition of TME in environment compared with natural sources. Due to heavy industry activities such as mining and smelting of nonferrous metals, transport, waste incineration, application of fertilizers, sewage sludge and communal waste based compost in agriculture, many sites have been polluted with excess of TME (Kabata-Pendias & Pendias, 2001). Constantly increasing contamination of many sites with high levels of TME poses a great risk to the public health and the environment (Aarts, 2012; Clemens et al., 2013b; Kabata-Pendias & Pendias, 2001).

3.2. Cadmium

Cadmium (Cd) is considered as one of the most toxic TME and a most serious health risk, due to its easy movement through the food chain. Cd does not play any role in the development of plants and is considered as non-essential element, however it is easily taken up by the root and leaf systems (Kabata-Pendias & Pendias, 2001; Nicholson et al., 2003; Kabata-Pendias, 2004; Willems et al., 2007; Robinson et al., 2009; Appenroth, 2010; Lin & Aarts, 2012; Clemens et al., 2013b; Verbruggen and Hermans, 2013; Małkowski et al., 2019). Accumulation of Cd in plants is in most cases proportional to the concentration of Cd in the soil or in the growth medium. Inside the plant Cd is transported more easily than other toxic metal elements such as Pb and Cr, however, it is still mainly accumulated in the roots (Kabata-Pendias & Pendias, 2001; Aarts, 2012; Małkowski et al., 2019.). Very high toxicity of Cd is connected with its high solubility in water and high mobility in the environment (Aarts et al., 2012). Additionally Cd had been reported to be highly reactive once inside the plant cells, especially with molecules containing thiol groups like for example amino acids (Huguet et al., 2012; Meng et al., 2019). Toxic effect of Cd on plants is also connected with the increase of oxidative stress levels by both direct stimulation of reactive oxygen species (ROS) generation as well as damaging of enzymes involved in ROS scavenging (Zawoznik et al., 2007; Das & Roychoudhury, 2014).

Although toxic effects of Cd have been reported for many biological systems, the mechanisms of Cd toxicity in plants are still not fully understood (Aarts et al., 2012; Clemens et al., 2013b; Ismael et al., 2019; Carvalho et al., 2020). Cd can either directly or indirectly exert a toxic effect on plant growth and development, various elements of photosynthesis process, biosynthesis of pigments, stomatal conductance, permeability of cell membranes and many other biological processes. Cd can also influence the uptake of other elements that are essential for plants (Kabata-Pendias & Pendias, 2001; Appenroth, 2010; Lin & Aarts, 2012; Kabata-Pendias & Szteke, 2015; Małkowski et al., 2019). Due to the geochemistry of Cd being strongly associated with Zn, the Cd-Zn interaction is one of the most commonly observed and studied in plants. (Kabata-Pendias & Pendias, 2001; Lin & Aarts, 2012; Clemens et al., 2013b; Kabata-Pendias & Szteke, 2015).

3.3. Zinc

Contrary to Cd, Zinc (Zn) is a micronutrient that plays essential roles in plants (Kabata-Pendias & Pendias, 1999, 2001; Broadley et al., 2012; Andresen et al., 2018; Castillo-González et al., 2018; Ghori et al., 2019). Zn is easily taken up by the plants and its accumulation in plants have been reported to be linear and proportional to its concentration in the soil (Kabata-Pendias & Pendias, 2001; Andresen et al., 2018). Zn is a component of various enzymes involved in metabolism of proteins, carbohydrates and phosphate. Zn is also linked with metabolism of auxins and formation of ribosomes and RNA (Kabata-Pendias & Pendias, 2001; Aarts et al., 2012; Kaur & Garg, 2021) Zn is also an essential structural element of proteins such as Zinc fingers, where Zn^{2+} helps to stabilize the structure of curls, which contact the major and minor grooves of the DNA. Moreover, as a structural element of superoxide dismutase (SOD), Zn is essential in positioning of the Cu atom to be accessible by the substrate (Castillo-González et al., 2018; Kaur & Garg, 2021).

Despite the essential role of Zn in plants, the excess concentration of this TME can have similar negative effect as Cd on different biological processes in plants. For example, excess Zn can cause a toxic effect on biosynthesis of pigments, respiration and different elements of photosynthetic apparatus such as light capture, electron transport and activity of Calvin cycle enzymes (Kabata-Pendias & Pendias, 2001; Aarts et al., 2012; Ghori et al., 2019). Moreover, it has been reported that excess Zn can disturb nitrogen metabolism and can decrease relative water content causing draught stress. Additionally Zn being a non-redox metal cannot directly

generate ROS, however at an excess it can disturb various metabolic pathways and electron transport mechanisms leading to induction of oxidative stress (Kaur & Garg, 2021).

3.4. Metallophytes

Soils with high levels of TME are very toxic to the vast majority of plant species. However, some plant species naturally acquired, during the course of evolution, the ability to colonize and thrive on heavily contaminated sites. These plant species, which are able to grow on metalliferous sites, are called metallophytes (Pollard et al., 2002, 2014; Krämer, 2010; Cappa & Pilon, 2013; van der Ent et al., 2013). Some of the species that adapted to metal contaminated sites can be described as pseudo-metallophytes or facultative metallophytes. Pseudo-metallophytes are plant species that have both metallicolous and non-metallicolous populations. Due to existence of two edaphic types, which are exposed to highly variable and distinct environmental condition, pseudo-metallophytes became a very important model for studying of plant local adaptations to TMEs contamination (Pollard et al., 2002; Maestri et al., 2010; Krämer, 2010; Verbruggen et al., 2013a).

3.5. Phenomenon of hyperaccumulation

Vast majority of plant species found on metalliferous soils have adapted to high TME contents by restriction of the uptake of metals by the roots and/or the limitation of their translocation to the shoots. In contrast some plant species have been found to have the ability to accumulate extremely high concentrations of TMEs in their aerial parts without exhibiting any symptoms of toxicity. These hyperaccumulator plant species are capable of accumulating high concentrations of trace metals (Zn, Ni, Mn, Cu, Co and Cd) or metalloids (As, Se) in their above ground parts (Pollard et al., 2002, 2014; Maestri et al., 2010; Krämer, 2010; Cappa & Pilon, 2013; van der Ent et al., 2013; Balafrej et al., 2020). Currently, for plant species to be considered as hyperaccumulator, the concentration threshold of element in μg per g of dry leaf tissue collected from plants growing in its natural habitat must exceed: 100 for Cd; 300 for Co and Cu; 1000 for As and Ni; 3000 for Zn and 10000 for Mn (van der Ent et al., 2013; Pollard et al., 2014; Goolsby et al., 2015).

Hyperaccumulation phenomenon attracted a lot of interest due to potential application in phytoremediation and phytomining of polluted environment as well as biofortification of crops (Yang et al., 2005; Robinson et al., 2009; van der Ent et al., 2015; Corso & García De La Torre, 2020). Hyperaccumulation is a rare phenomenon and so far only around 720 plant species

have been identified as hyperaccumulators of TME, with most of them being hyperaccumulators of Ni. Majority of identified hyperaccumulator species have been found to be obligatory metallophytes found growing only on metalliferous soils (Krämer, 2010; van der Ent et al., 2013; Pollard et al., 2014; Goolsby et al., 2015; Reeves et al., 2018). However, some hyperaccumulating plant species with both metallicolous and non-metallicolous populations have been found to be an exceptional models for better understanding of mechanisms of TME homeostasis in plants, as well as their adaptation to metalliferous environment. One species, which is considered as model in such studies, is the pseudometallophyte and hyperaccumulator of Cd and Zn *Arabidopsis halleri* (Küpper et al., 2000; Roosens et al., 2008; Verbruggen et al., 2009b; van der Ent et al., 2015; Sitko et al., 2017; Schvartzman et al., 2018).

3.6. *Arabidopsis halleri*

Arabidopsis halleri, previously known as *Cardaminopsis halleri* is a self-incompatible perennial herb, which is widespread across Europe and Eastern Asia. Populations of *A. halleri* can be found around forest margins, meadows and rocky slopes (Moulon et al., 2005; Clauss & Koch, 2006; Fiałkiewicz & Rostański, 2006; Hunter & Bomblies, 2010; Godé et al., 2012; Wasowicz et al., 2016; Sitko et al., 2017; Stein et al., 2017; Frérot et al., 2017).

Fig.1 *A. halleri* in the metalliferous site in Piekary Śląskie.

A. halleri is a pseudometallophyte species that have been identified as a hyperaccumulator of Cd and Zn. While Zn hyperaccumulation is constitutive this species, Cd hyperaccumulation is more variable. Due to variability within the species with various edaphic types it have been used in many studies regarding the phenomenon of hyperaccumulation and hypertolerance in plants (Bert et al., 2003; Weber et al., 2004; Baliardini et al., 2015; Isaure et al., 2015; Meyer et al., 2015; Sitko et al., 2017; Stein et al., 2017; Corso et al., 2018; Schvartzman et al., 2018; Szopiński et al., 2019). As a member of *Brassicaceae* family *A. halleri* is closely related to the model species *Arabidopsis thaliana*, widely used in molecular

research due to relatively small genome size. The genome of *A. halleri* is approximately 40-60% larger than that of *A. thaliana* at around 0.56 pg, with 2*n* = 16 chromosomes. Especially useful for researchers turned out to be a fact that *A. halleri* shares 94% similarity of nucleotide-sequence within coding regions with *A. thaliana* (Godé et al., 2012; Kolník & Marhold, 2006; Roosens et al., 2008). This has allowed for the molecular data from *A. thaliana* to be used in order to identify genes with different expression profiles underlying Cd and Zn hyperaccumulation and hypertolerance in *A. halleri* (Weber et al., 2004; Roosens et al., 2008; Verbruggen et al., 2009b).

Within *A. halleri* species hyperaccumulation and tolerance to Cd and Zn varies between populations from both metalliferous and non-metalliferous soils (Meyer et al., 2010; Stein et al., 2017; Corso et al., 2018). Investigations showed that *A. halleri* appeared first on non-metalliferous soils. In the course of evolution populations of *A. halleri* acquired the ability to colonize metalliferous sites. However, some recent studies documented that some non-metallicolous populations found in Europe originated from metallicolous populations (Babst-Kostecka et al., 2018).

3.7. *Arabidopsis arenosa*

Arabidopsis arenosa (Previously known as *Cardaminopsis arenosa*) is another member of *Brassicaceae* family related with model species *A. thaliana*. It is a perennial herb, which is widespread throughout Europe. *A. arenosa* can commonly be found in exposed grassy and sandy habitats such as railroad tracks, roadsides, river banks and forest margins (Przedpełska & Wierzbicka, 2007; Hunter & Bomblies, 2010; Przedpelska-Wasowicz & Wasowicz, 2013; Wright et al., 2015; Gieroń e al., 2021a). Despite this species being so widespread and frequently observed, few studies have used *A. arenosa* compared with other species from Arabidopsis genus such as *A. thaliana* and *A. halleri* (Clauss & Koch, 2006;

Fig.2 *A. arenosa* in the native metalliferous site in Piekary Śląskie.

Staňová et al., 2012; Turisová et al., 2013; Baduel et al., 2016; Rozpądek et al., 2018; Szopiński et al., 2019; Gieroń, Sitko, Zieleźnik-Rusinowska, et al., 2021).

A. arenosa is a ploidy variable plant species with majority of populations reported so far being tetraploid with 2*n*=4*X*=32 and some being diploid with 2*n* = 2*X* = 16 (Hollister et al., 2012; Schmickl et al., 2012; Yant et al., 2013; Higgins et al., 2014; Gieroń et al., 2021b). Tetraploid populations are mostly distributed through central and northern Europe, whereas diploid populations are mostly found in eastern Europe and the Balkans (Schmickl et al., 2012; Yant et al., 2013; Arnold et al., 2015; Kolář et al., 2016). Genome size of *A. arenosa* is approximately 150% larger, due to genome duplication, than that of its *A. thaliana* relative. Despite being a part of *Arabidopsis* genus, *A. arenosa* is related more closely with *A. halleri* compared with *A. thaliana* (Novikova et al., 2016). *A. arenosa* have been mainly used in molecular studies exploring the phenomenon of polyploidization in plants (Clauss & Koch, 2006; Hollister et al., 2012; Yant et al., 2013; Del Pozo & Ramirez-Parra, 2015; Hollister, 2015).

A. arenosa have been commonly reported from habitats contaminated with TMEs (Rostański et al., 2005; Clauss & Koch, 2006; Szarek-Łukaszewska & Grodzińska, 2007; Szarek-Lukaszewska & Grodzińska, 2011; Turisová et al., 2013; Borymski et al., 2018; Gieroń, et al., 2021b). Despite common occurrence of *A. arenosa* on TME contaminated sites, this species did not get as much attention as its close relative hyperaccumulator of Cd and Zn *A. halleri*. Some studies, however, mention *A. arenosa* in the context of tolerance to TME such as Cd and Zn (Przedpełska & Wierzbicka, 2007; Nadgórska-Socha et al., 2013; Przedpelska-Wasowicz & Wasowicz, 2013; Baduel et al., 2016; Szopiński et al., 2019, 2020; Gieroń, et al., 2021b). Moreover, in recent time, more focus have been put in to studying the evolutionary history of both *A. arenosa* and *A. halleri* (Preite et al., 2019).

3.8. Mechanisms of Cd and Zn hyperaccumulation

Molecular mechanisms of uptake and transport of essential microelements in plants have been under investigation for a very long time. Cd is not essential and there are no dedicated transport systems for it in plants. However, due to similar physicochemical properties as other divalent ions such as Zn, Fe and Mn, Cd competes at the catalytic sites of non-specific transporters (Verbruggen et al., 2009b; Balafrej et al., 2020; Corso & García De La Torre, 2020;

Luo & Zhang, 2021). Hyperaccumulators can be distinguished from non-hyperaccumulator species by enhanced rate of TME uptake, highly efficient translocation of TME from roots to shoots and ability to tolerate and detoxify high concentration of TME in leaves (Krämer et al., 2007; Verbruggen et al., 2009b; Rascio & Navari-Izzo, 2011; Aarts et al., 2012; Li et al., 2018; Sytar et al., 2020). Molecular studies investigating hyperaccumulator and related non-hyperaccumulator plant species found that key elements of mechanisms underlying phenomenon of hyperaccumulation and hypertolerance can be attributed to different regulation and overexpression of genes encoding transmembrane transporters. Among the transporter families identified as the ones playing a key role in this phenomenon are ZIP (Zinc-regulated transporter, Iron-regulated transporter protein), NRAMP (Natural resistance associated macrophage), MATE (Multidrug and toxin efflux), OPT (Oligopeptide transporter), MTP (Metal tolerance protein), CAX (Cation exchanger) and P-type metal ATPases (Krämer et al., 2007; Verbruggen et al., 2009b; Rascio & Navari-Izzo, 2011; Balafrej et al., 2020; Corso & García De La Torre, 2020; Luo & Zhang, 2021). Transporters involved in hyperaccumulation can be divided into four main groups responsible for: uptake, transport and translocation and detoxification. The localisation and the proposed role in Zn and Cd hyperaccumulation of the transporters encoded by the genes analysed in my project are presented on Fig. 3.

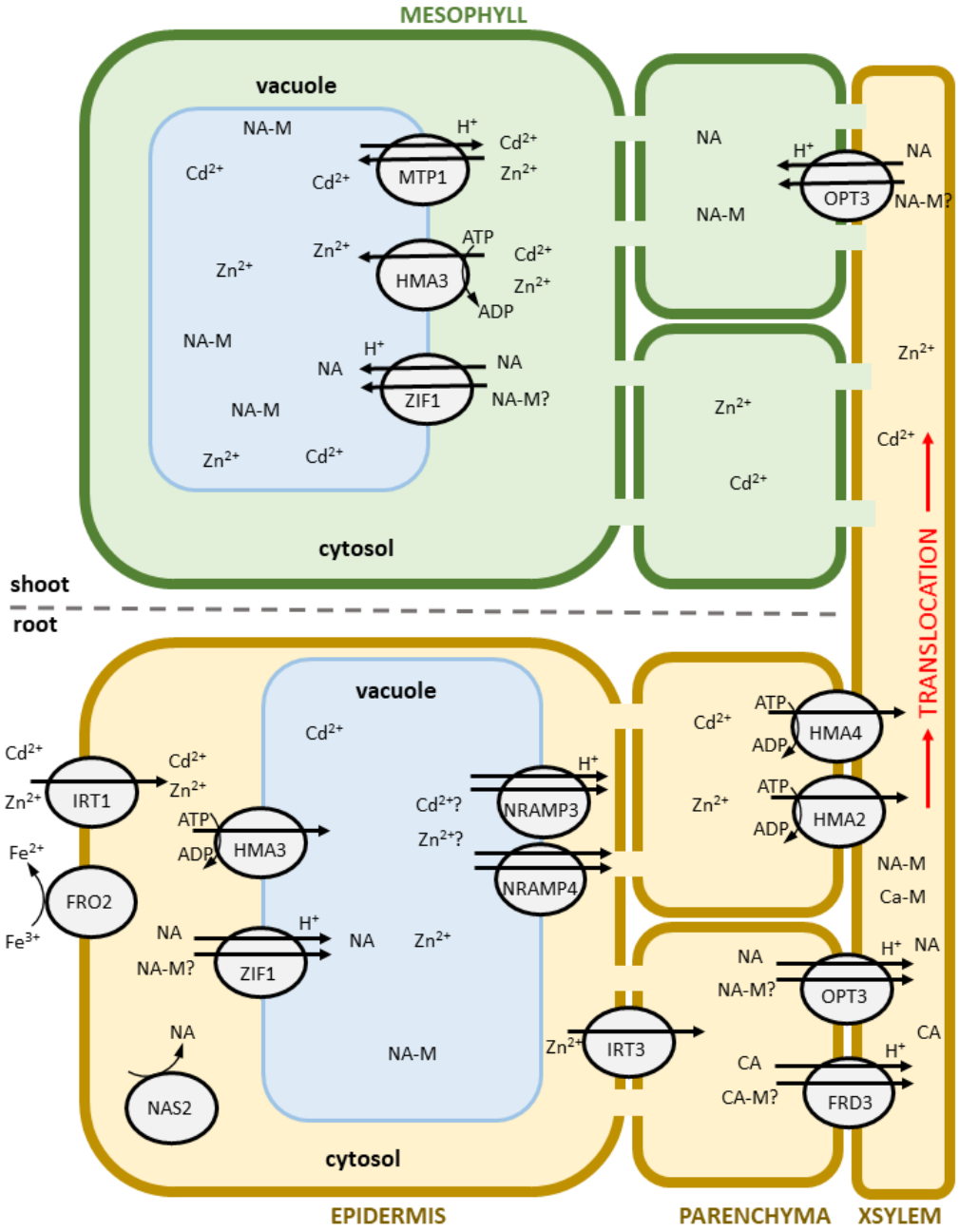

Figure 3. Role and localisation of transporters involved in Cd and Zn uptake, transport and detoxification investigated in the current study. Sitko (2019), modified. Each transporter is described in text below.

3.8.1. Cd and Zn uptake

Cd and Zn are taken up by the roots in the form of divalent cations (Cd^{2+} and Zn^{2+}) by the transmembrane transporters from ZIP family. Members of ZIP family are involved in the transport of metal ions, such as Fe^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} as well as Cd^{2+}. IRT1, an Fe and Zn regulated transporter was shown to mediate Fe, Zn and Cd uptake in *A. thaliana* and is

speculated to also contribute to Fe as well as Zn and Cd accumulation in *A. halleri* (Shanmugam et al., 2011; Dubeaux et al., 2018; Corso et al., 2021). On the other hand, IRT3 have been found to be able to transport Fe^{2+} and Zn^{2+} but not Cd^{2+} inside the root of *A. thaliana* and *A. halleri* and is involved in the cellular uptake of these metals(Guerinot, 2000; Krämer et al., 2007; Lin et al., 2009; Verbruggen et al., 2009b; Hanikenne & Nouet, 2011; Andresen et al., 2018). Moreover, ZIP4, ZIP5, ZIP6, ZIP9, ZIP10, ZIP11 transporters that had been found highly expressed in metal hyperacumulating species showed ability to transport Zn, however, only ZIP4, ZIP5 and ZIP9 were proposed to also be able to transport Cd (Talke et al., 2006; Van De Mortel et al., 2006; Feng et al., 2016; Peng et al., 2017; Corso & García De La Torre, 2020). Additionally, plasma membrane NRAMP1 symporter have been found to play a role in Fe^{2+} and Mn^{2+} uptake in *A. thaliana* and was also proposed to be involved in Cd^{2+} and Zn^{2+} uptake from the growth media (Aarts et al., 2012; Milner et al., 2014; Bian et al., 2018).

3.8.2. Cd and Zn transport and translocation

While non-hyperaccumulator species mainly sequester toxic metals in vacuoles of root cells, in hyperaccumulators excess of Cd^{2+} and Zn^{2+} after being sequestered in the vacuole must be efficiently remobilised. Tonoplast symporters like NRAMP3 and NRAMP4 involved in remobilisation of Fe^{2+} and Mn^{2+} from the vacuoles, contribute to Zn and Cd tolerance by mediating appropriate Fe and Mn supply (Molins et al. 2013). NRAMP3 and NRAMP4 had also been proposed to contribute to Cd and Zn remobilisation in *A. halleri* (Krämer et al., 2007; Verbruggen et al., 2009b; Rascio & Navari-Izzo, 2011; Aarts et al., 2012; Andresen et al., 2018; Corso et al., 2021). Due to this remobilization from vacuole Cd^{2+} and Zn^{2+} can easily efflux and be loaded into the xylem and translocated into the shoots. P_{1B}-type metal ATPases, also known as heavy metal transporting ATPases (HMA) have been shown to play a major role in TME efflux from cell and loading into the xylem with the use of energy from ATP hydrolysis. In *A. halleri*, heavy metal pumps HMA2 and HMA4 play important role in Cd^{2+}/Zn^{2+} efflux from the cells and loading into the xylem. Especially HMA4 is described as the main driving force behind hyperaccumulation due to the triplication and constitutive overexpression of *HMA4* of this gene in the *A. halleri* (Krämer et al., 2007; Verbruggen et al., 2009b; Hanikenne & Nouet, 2011; Rascio & Navari-Izzo, 2011; Park & Ahn, 2014; Nouet et al., 2015). Studies showed that in the xylem sap of *A. halleri* Cd is transported mainly in free ionic form (Ueno et al., 2008). However, it have been reported that both Cd and Zn may also be transported in the form of

chelates with organic compounds such as citrate and nicotianamine (NA) (Bauer & Schuler, 2011). Citrate is transported by Ferric Reductase Defective 3 transporter (FRD3), a member of MATE family. FRD3 in *A. thaliana* and *A. halleri* was shown to play important role in Fe and Zn homeostasis and is suggested to be able to transport metals chelated with citrate (Roschzttardtz et al., 2011; Charlier et al., 2015; Scheepers et al., 2019; Corso et al., 2021). Among the transporters suggested to be involved in the transport of NA metal chelates, are members of OPT family like OPT3, YSL (Yellow stripe-like) subfamily like YSL3 (Verbruggen et al., 2009b; Hanikenne & Nouet, 2011; Rascio & Navari-Izzo, 2011; Zhai et al., 2014; Andresen et al., 2018; Schvartzman et al., 2018; Kim et al., 2021).

3.8.3. Cd and Zn detoxification

Once inside the leaf cells, Cd^{2+} and Zn^{2+} are actively sequestered inside the vacuole of *A. halleri* by another member of P-type metal ATPases, namely HMA3 (Krämer et al., 2007; Verbruggen et al., 2009b; Hanikenne & Nouet, 2011; Rascio & Navari-Izzo, 2011; Park & Ahn, 2014; Mishra et al.,2017). Moreover, members of CAX family, a tonoplast antiporters such as CAX2 and CAX4 have a wide range of substrate specificity and also play a role in sequestration of Cd and Zn ions in the vacuole of *A. halleri* (Mei et al., 2009; Verbruggen et al., 2009b; Pittman & Hirschi, 2016; Andresen et al., 2018; Corso et al., 2018). Furthermore, transporters like MTP1 play a role in transport of Zn ions into the vacuole (Talke et al., 2006; Verbruggen et al., 2009b; Rascio & Navari-Izzo, 2011; Küpper & Andresen, 2016; Fasani et al., 2017; Andresen et al., 2018). In addition, detoxification of Cd and excess Zn takes place through their chelation with organic compounds such as organic acids, flavonoids, amino acids, peptides and proteins. High expression of nicotianamine synthetase (NAS) genes together with increased content of nicotianamine was observed in *A. halleri*. Zinc-induced facilitator 1 (ZIF1) responsible for NA transport into the vacuole was also found to be important for Zn tolerance in *A. thaliana* (Haydon et al., 2012). Moreover, ABC transporters (ATP-binding cassette) with the use of energy from ATP hydrolysis. However the role of transporters from ABC family in hyperaccumulation mechanisms are still poorly understood (Rea, 2007; Verbruggen et al., 2009b; Rascio & Navari-Izzo, 2011; Do et al., 2018; Zhang et al., 2018).

Flavonoids are the largest and highly diverse group of polyphenolic secondary plant metabolites with low molecular weight. Flavonoids occur in plants mainly in the form of O-glycosides. They are characterised by two benzene rings that are linked together by a

heterocyclic pyran ring (Mierziak et al., 2014; Shah & Smith, 2020). Flavonoids are synthesised in all parts of the plant and are responsible for aroma, taste and colour of flowers, fruits and seeds, which in turn attracts pollinators. They have been also proposed to play a vast array of biological functions in plants responses to abiotic (heat, UV radiation, TME) and biotic (pathogens, herbivores) stress factors (Winkel-Shirley, 2002; Agati et al., 2012, 2013). This functional diversity can be attributed to high structural variance that is increased by further modifications (e.g. methylation, glycosylation, phosphorylation etc.). Amongst most notable flavonoid classes that have been implicated in being involved in tolerance to TME are flavonols and anthocyanins (Winkel-Shirley, 2002; Corso et al., 2018; Schvartzman et al., 2018; Shah & Smith, 2020). Flavonols are the largest group of flavonoids in plants, with quercetin (Fig.4a) being one of the most studied compounds in this group. Whereas cyanidin (Fig. 4b) being one of the most common anthocyanins (Keilig & Ludwig-Müller, 2009; Shah & Smith, 2020). Flavonols and anthocyanins play an important role in plants as protection against excessive

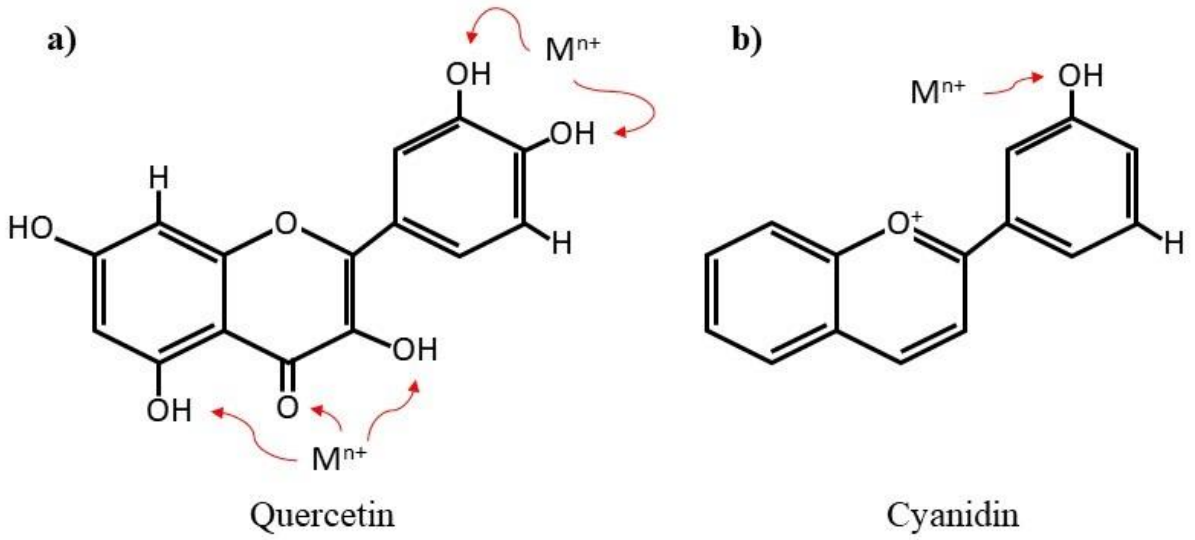

Fig.4 Skeleton structure of Quercetin (a) and Cyanidin (b) with possible metal chelation sites marked (red arrows). M^{n+} - divalent or trivalent metal cation.

light exposure and UV radiation. Moreover they are involved in defence against damage caused by TME toxicity. They protect plants from damage through scavenging of ROS, quenching of lipid peroxidation reactions and by direct chelating of toxic metals such as Cd (Keilig & Ludwig-Müller, 2009; Nakabayashi et al., 2014; Asad et al., 2015; Xu & Rothstein, 2018; Shah & Smith, 2020).

Phytochelatins (PCs) glutathione (GSH) and metallothioneins (MTs) are another important element of TME detoxification in plants. MTs are synthesised on the ribosomes, while PCs and GSH are synthesised enzymatically in the cytosol. These small non-protein peptides are responsible for maintenance of redox balance in plant cells as well as direct chelation of toxic metal ions thanks to the presence of thiol groups (Pal & Rai, 2010; Das & Roychoudhury, 2014; Luo & Zhang, 2021). Currently suggested involvement of these peptides in the mechanism of TME detoxification is formation of complexes with toxic metals, which are in turn sequestered into the vacuoles by the ABC transporters (Pal & Rai, 2010; Luo & Zhang, 2021).

4. OBJECTIVES OF THE PhD

This PhD project aims at improving the knowledge of pseudometallophytes, in particular of two species closely related to the model species *Arabidopsis thaliana*, which are present on both metalliferous and non-metalliferous soils in Poland: *Arabidopsis halleri* and *Arabidopsis arenosa*.

Our specific aims address the following questions:

1. Does *A. arenosa* adapt the same way to contaminated environment as *A. halleri in situ* and in controlled conditions?

2. Does *A. arenosa* metallicolous population respond the same way as *A. halleri* metallicolous population under short term treatment with high Cd and Zn concentrations?

3. Are there differences in the mechanisms of metal homeostasis between *A. halleri* and *A. arenosa*. In particular can we observe the differences in the expression of genes involved in metal uptake, translocation and detoxification in metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa* in response to Cd and Zn treatments?

5. SCIENTIFIC STRATEGY

In order to address the scientific questions put forward, experiments were carried out on *A. halleri*, a model species for study of TMEs homeostasis mechanisms and closely related species *A. arenosa.* Due to the interdisciplinary nature of the research the project was done under the joint supervision between University of Silesia in Katowice (UŚ) represented by prof. dr hab. Eugeniusz Małkowski and Université libre de Bruxelles (ULB) represented by prof. Nathalie Verbruggen. Physiological part of the project was carried out in Plant Ecophysiology Team (Institute of Biology, Biotechnology and Environmental Protection, UŚ). The gene

expression analysis was performed in the Laboratory of Plant Physiology and Molecular Genetics (ULB).

The doctoral thesis included the following tasks:

1. Investigation of physiological parameters of *A. halleri* and *A. arenosa* populations growing in the TMEs contaminated as well as non-metalliferous sites. During the investigation following analyses were performed:

   a) Measurements of Ca, Cd, Mg, Fe, Mn, Pb, Zn, and Cu content in the soil as well as in the roots and shoots of all populations.

   b) Analysis of photosynthetic apparatus activity on the basis of chlorophyll *a* fluorescence measurements.

   c) Measurements of chlorophyll, flavonol and anthocyanin content.

   d) Determination of DNA content and ploidy level using flow cytometry.

2. Investigation of physiological parameters of *A. halleri* and *A. arenosa* metallicolous and non-metallicolous populations treated with various concentrations of Cd in hydroponic conditions. The specific tasks included:

   a) Measurements of Ca, Cd, Mg, Fe, Mn, Pb, Zn, and Cu in roots and shoots.

   b) Measurements of chlorophyll *a* fluorescence to assess the photosynthetic apparatus activity.

   c) Measurements of chlorophyll, flavonol and anthocyanin content.

3. Characterisation of physiological responses of metallicolous population of *A. halleri* and *A. arenosa* to high Cd and Zn treatments. Task included:

   a) Measurements of Cd and Zn in shoots.

   b) Analysis of photosynthetic apparatus tolerance based on chlorophyll *a* fluorescence measurements.

   c) Measurements of chlorophyll, flavonol and anthocyanin content.

   d) Measurements of oxidative stress level indicators.

4. Investigation of differences in expression of genes involved in TMEs uptake, translocation and detoxification between metallicolous and non metallicolous populations of *A. halleri* and *A. arenosa* under Cd and Zn treatment. As part of the investigation the specific tasks were:

   a) Determination of Cd and Zn tolerance class of all studied populations, through incrementally increasing concentrations.

b) Measurements of Ca, Cd, Mg, Fe, Mn, Pb, Zn, and Cu in roots and shoots.

c) Measurements of chlorophyll, flavonol and anthocyanin content.

d) Measurements of expression levels of *HMA2*, *HMA3*, *HMA4*, *MTP1*, *IRT1*, *IRT3*, *FRD3*, *FRO2*, *ZIF1*, *NRAMP3*, *NRAMP4*, *OPT3*, *NAS2*, *LDOX* and *F3H* genes with the use of Real-Time quantitative PCR method.

e) Analysis of *HMA4* gene copy number in all investigated populations.

To compare the two Arabidopsis species, we have evaluated their levels of tolerance, TMEs accumulation, the activity of photosynthetic apparatus, pigments accumulation as well as the profile of expression of genes involved in TMEs homeostasis of metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa* growing in their native sites and controlled hydroponic conditions under TMEs stress. As a result, the following three papers were prepared to respectively answer the three scientific questions:

1. Characterisation of TMEs accumulation, photosynthetic apparatus parameters and pigments content of metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa in situ* and in hydroponic cultures under Cd treatments. - **CHAPTER I**

2. Evaluation of Cd and Zn accumulation, photosynthetic apparatus activity, level of oxidative stress and pigments content in metallicolous population of *A. halleri* and *A. arenosa* under short term treatment with high Cd and Zn concentration. - **CHAPTER II**

3. Analysis of differences in expression level of genes underlying mechanisms of metal uptake, translocation and detoxification in metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa* in response to Cd and Zn treatment. - **CHAPTER III**

6. METHODS USED

The methods used are described in each results chapter. We briefly introduce them below. However, due to space limitation in the publications, the analysis of photosynthetic activity using chlorophyll a fluorescence was not sufficiently described and is presented here in more details.

6.1. Chlorophyll *a* fluorescence analysis

Illumination of plants with light leads to the emission of some of the absorbed energy back as fluorescence in the range of wavelength of 660-780 nm. The main source of this fluorescence is excited chlorophyll *a* (Chl *a*) molecules in the light-harvesting antenna that is connected with photosystem II (PSII) (Baker, 2008; Goltsev et al., 2009; Kalaji et al., 2012; Stirbet et al., 1998). Emission of fluorescence by excited Chl *a* induced by photosynthetically active light is known as Kautsky effect or the fluorescence transient. At the basis of fluorescence transient analysis lies the state of reaction centers of PSII, which is generally accepted to be defined by the redox state of primary quinone electron acceptor, namely plastoquinone A (Q_A). In general, when Q_A in reaction center is in reduced state (Q_A^-), emitted fluorescence is high and the reaction center is considered to be closed. In contrast when Q_A is in oxidized state, the fluorescence is minimal and reaction center is considered to be open (Stirbet et al., 1998; Baker, 2008; Goltsev et al., 2009; Sitko et al., 2017). Measurements of Chl *a* fluorescence transient are performed on 30 min dark-adapted leaves that are illuminated with a flash of saturating light that induces the reduction of Q_A over the time of 1s when the fluorescence reaches its maximum (Kalaji et al., 2012, 2014, 2018; Sitko et al., 2017). Typical Chl *a* fluorescence induction transients, otherwise known as OJIP test is curve on which four distinctive points/peaks can be identified: minimal fluorescence at the start of measurement (F_0 or O), J (around t = 0.001 s), I (around t = 0.01 s) and maximal fluorescence (F_m or P). First O-J phase of fluorescence transient is strongly light-dependent and can be useful for identifying the size of the antenna and the connectivity between reaction centers. O-J phase is also strongly influenced by the state of the electron donors inside PSII. While second I-P phase, otherwise known as thermal phase carries information about the size of plastoquinone pool (PQ) and redox state of reaction centers reducing it, as well as connectivity with PSI and the state of end elements of chloroplast electron transport chain, such as Ferredoxin-$NADP^+$ Reductase (FNR). Moreover, outside of typical

points, few distinct intermediate bands can also be identified as K (around 0.0002 s), H (around 0.02 s) and G (around 0.1 s) (Schansker et al., 2006; Kalaji et al., 2012; Paunov et al., 2018; Szopiński et al., 2019). Changes in redox state of elements of electron transport chain in chloroplasts are reflected on Chl *a* fluorescence transient as distinctive peaks and intermediate bands and are presented on Fig. 1.

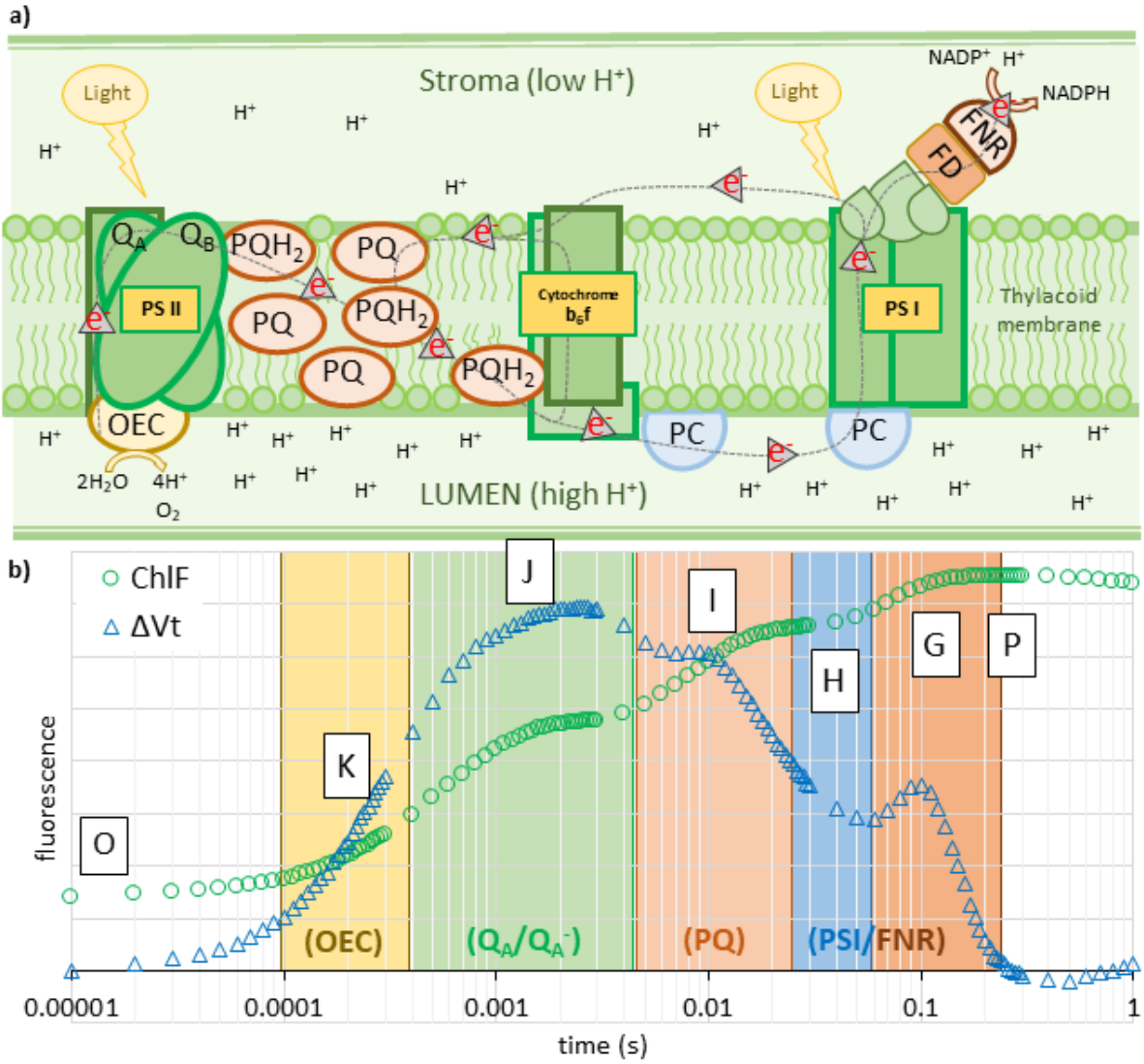

Fig.1 Diagram of electron transport chain through the thylakoid membrane (a) and its translation into the curves of chlorophyll *a* fluorescence (ChlF) and variable relative fluorescence (ΔVt) (b). Possible electron transport routes along with the directions are marked with a grey line. List of abbreviations: OEC - Oxygen Evolving Complex; QA and QB - quinone electron acceptors in PSII; PQ - plastoquinone; PC - plastocyanin; FD for ferredoxin; FNR - ferredoxin-NADP $^{+}$ oxidoreductase; O, K, J, I, H, G, P - characteristic peaks for the fluorescence curve, more widely discussed in the text. Sitko (2019), modified.

Whilst standard OJIP test is very informative, having a control group in your experiment allows mathematical transformation of variable fluorescence into variable relative fluorescence

(ΔV_t) by subtracting the fluorescence value of control from the one measured for experimental variant. Such transformation allows clear comparison of the state of elements of electron transport chain reflected by distinct elements of OJIP test (ΔK, ΔJ, ΔI, ΔH and ΔG) in each experimental variant (Fig. 1). Presence of positive ΔK band is usually associated with damage or inactivation of Oxygen Evolving Complex (OEC). ΔJ peak reflects the accumulation of Q_A^- caused by reduced capability of reaction centers of PSII to re-oxidize it. Peak ΔI relates to reduced electron transfer into the b_6f cytochrome, that is usually connected with reduced pool of PQ electron transporter. ΔH and ΔG points are associated with the activity of end electron acceptors of PSI such as FNR (Stirbet et al., 1998; Schansker et al., 2006; Kalaji et al., 2012, 2014; Sitko et al., 2017; Paunov et al., 2018; Szopiński et al., 2019).

6.2. Mineral Analysis

Mineral analysis was performed using flame atomic absorption spectrophotometry (iCE 3,500 FAAS, Thermo Scientific) on soil and plant samples, that had been acid digested in the microwave-assisted wet digestion system (ETHOS 1, Milestone, Italy).

6.3. Pigments measurement

The measurements of chlorophyll, flavonol and anthocyanin content indices were taken using a fluorimeter (Dualex Scientific+ sensor, Force-A, France).

6.4. Gas-Exchange Parameters

The measurements of gas-exchange parameters were performed using an infrared gas analyser with special chamber for Arabidopsis (LCpro+, ADC Bioscientific, UK) under controlled climate conditions (T = 24°C, Ambient light PAR = 150 μmol m^{-2} s^{-1}).

6.5. Ploidy level analysis

The content of DNA was measured using Space flow cytometer (CyFlow, Sysmex, Kobe, Japan) with a 365-nm UV LED diode as the light source.

6.6. Gene expression analysis

Reactions to analyse the level of gene expression were prepared with SYBR Green mastermix (Promega, Fitchburg, WI, USA). Analysis was done using the Real-time PCR method in 96-well plates with the PikoReal real time PCR system (Thermo Scientific, Loughborough, 519 UK).

6.6. Statistical analysis

The statistically significant differences among the mean values were determined using a one-way ANOVA and a *post hoc* HSD test ($P < 0.05$) (Chapters I and II) or LSD test ($P < 0.05$) (Chapter III). The statistical analysis was performed using Statistica v.13.1 software (Dell Inc.).

7. CHAPTER I: Different strategies of Cd tolerance and accumulation in *Arabidopsis halleri* and *Arabidopsis arenosa*

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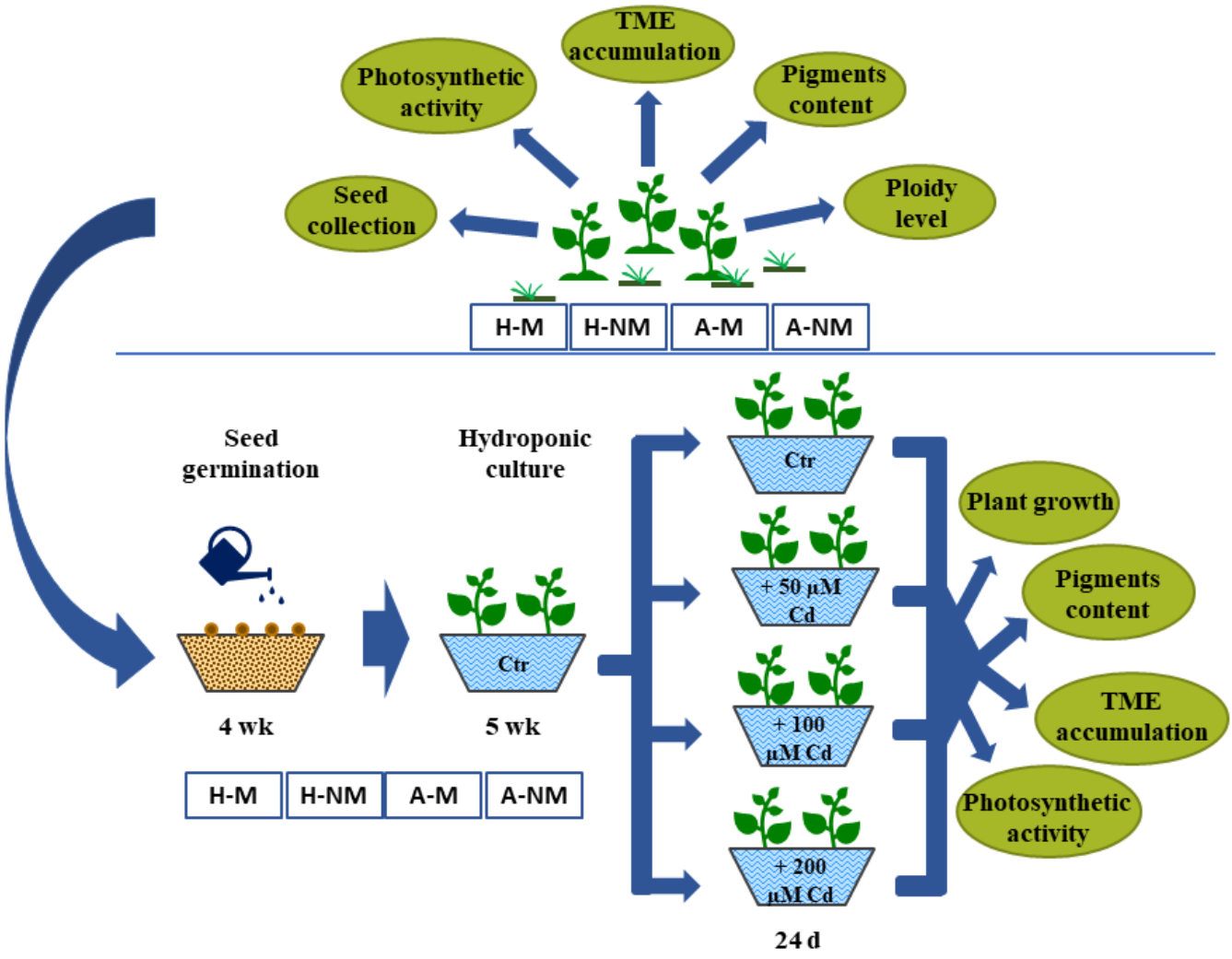

Graphical abstract

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ORIGINAL ARTICLE

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Different strategies of Cd tolerance and accumulation in *Arabidopsis halleri* and *Arabidopsis arenosa*

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Abstract

Pseudometallophytes are commonly used to study the evolution of metal tolerance and accumulation traits in plants. Within the *Arabidopsis* genus, the adaptation of *Arabidopsis halleri* to metalliferous soils has been widely studied, which is not the case for the closely related species *Arabidopsis arenosa*. We performed an in-depth physiological comparison between the *A. halleri* and *A. arenosa* populations from the same polluted site, together with the geographically close non-metallicolous (NM) populations of both species. The ionomes, growth, photosynthetic parameters and pigment content were characterized in the plants that were growing on their native site and in a hydroponic culture under Cd treatments. *In situ*, the metallicolous (M) populations of both species hyperaccumulated Cd and Zn. The NM population of *A. halleri* hyperaccumulated Cd and Zn while the NM *A. arenosa* did not. In the hydroponic experiments, the NM populations of both species accumulated more Cd in their shoots than the M populations. Our research suggests that the two *Arabidopsis* species evolved different strategies of adaptation to extreme metallic environments that involve fine regulation of metal homeostasis, adjustment of the photosynthetic apparatus and accumulation of flavonols and anthocyanins.

KEYWORDS

Arabidopsis, cadmium, chlorophyll fluorescence, electron transport, heavy metals, hyperaccumulation, photosynthesis, photosystem II

1 | INTRODUCTION

As a result of human activities such as mining and the smelting of non-ferrous metals as well as natural processes (e.g., volcanic emissions, weathering and the erosion of soil, forest fires, etc.), many sites have been contaminated with trace metallic elements (TME) such as cadmium (Cd), lead (Pb), zinc (Zn) and copper (Cu). Soils that have been contaminated with very high concentrations of TME pose a threat to the environment and to public health. Cd is one of the most toxic elements and its accumulation in the soil can cause alterations in plant processes such as disrupting photosynthesis, competing with the essential elements and causing cellular damage (Clemens & Ma, 2016; Myśliwska-Kurdziel, Prasad, & Strzałka, 2002; Verbruggen, Juraniec, Balliardini, & Meyer, 2013). The plant species that grow on metalliferous sites are called metallophytes. Among them, pseudometallophytes, which are also called facultative metallophytes, are plant species that can be found on both metalliferous and non-metalliferous sites. Metallophytes and pseudometallophytes are powerful biological models that can be used to study the physiological and molecular mechanisms that are involved in the tolerance and accumulation of high concentrations of TME in plants (Bothe & Słomka, 2017; Corso et al., 2018; Milner & Kochian, 2008; Pollard, Reeves, & Baker, 2014; Schvartzman et al., 2018; Verbruggen, Hanikenne, & Clemens, 2013; Verbruggen, Juraniec, et al., 2013). The majority of metallophytes have developed mechanisms to exclude TME from their tissues. Hyperaccumulators belong to a rare class of plant species that are capable of accumulating extraordinarily high concentrations of these metals in their above-ground tissues without any visible symptoms of toxicity (Baker, McGrath, Reeves, & Smith, 2000; Clemens & Ma, 2016; Maestri, Marmiroli, Visioli, & Marmiroli, 2010; Pollard et al., 2014; Van der Ent, Baker, Reeves, Pollard, & Schat, 2013; Verbruggen, Hermans, & Schat, 2009; Verbruggen, Juraniec, et al., 2013). The threshold for Cd hyperaccumulation is 100 μg/g, while for Zn, it is 3,000 μg/g of the dry leaf tissue that is collected from plants that are growing in their natural habitat (Krämer, 2010; Pollard et al., 2014; Van der Ent et al., 2013; Van der Ent, Baker, Reeves, Pollard, & Schat, 2015).

Arabidopsis halleri is a pseudometallophyte that is closely related to *A. thaliana*, which has commonly been used to study the adaptations to extreme conditions that occur on metalliferous sites (Corso et al., 2018; Fiałkiewicz & Rostański, 2006; Krämer, 2010; Peng et al., 2020; Sitko, Rusinowski, Kalaji, Szopiński, & Małkowski, 2017; Verbruggen, Juraniec, et al., 2013). Phylogeographic studies have suggested that in most cases, non-metallicolous (NM) populations of *A. halleri* have developed the ability to inhabit nearby metalliferous sites over the course of their evolution (Al-Shehbaz & O'Kane, 2002; Baker et al., 2000; Hunter & Bomblies, 2010; Pauwels et al., 2012). However, Babst-Kostecka et al. (2018) recently showed that the NM metallicolous population in Poland have derived from metallicolous (M) populations.

While Zn hyperaccumulation seems to be a constitutive trait in *A. halleri*, Cd accumulation is highly variable (Frérot et al., 2018; Huguet et al., 2012; Meyer et al., 2015; Pauwels et al., 2012; Pollard et al., 2014; Sitko et al., 2017; Stein et al., 2017). Despite several recent studies, the mechanisms that underlie Cd hyperaccumulation are still not fully understood (Corso et al., 2018; Frérot et al., 2018; Mishra, Mishra, & Küpper, 2017; Sitko et al., 2017; Stein et al., 2017; Zhang, Wen, Huang, Inoue, & Liang, 2017).

Arabidopsis arenosa is a pseudometallophyte that is genetically closer to *A. halleri* than to *A. thaliana* and is widespread throughout Northern and Central Europe (Al-Shehbaz & O'Kane, 2002; Clauss & Koch, 2006; Hunter & Bomblies, 2010; Novikova et al., 2016; Rostański, Myśliwiec, & Siwińska, 2005; Turisová, Štraba, Aschenbrenner, & Andráš, 2013). Metallicolous populations of *A. arenosa* have been reported to be very tolerant to TME such as Cd and Zn (Nadgórska-Socha, Ptasiński, & Kita, 2013; Przedpełska & Wierzbicka, 2007; Szopiński et al., 2019). *Arabidopsis arenosa* has recently emerged as a model to study genome duplication and autopolyploidization. This plant species is ploidy-variable with both diploid and tetraploid populations that are unevenly distributed across Europe (Higgins, Wright, Bomblies, & Franklin, 2014; Hollister, 2015; Kolář et al., 2016; Lafon-Placette et al., 2017; Lloyd & Bomblies, 2016; Novikova, Hohmann, & Van de Peer, 2018; Yant et al., 2013). The literature that is available on *A. arenosa* is limited and only a few papers have described the physiology of this species in general (Bothe & Słomka, 2017; Clauss & Koch, 2006; Kenderešová et al., 2012). Although two studies documented the accumulation of Cd in the shoots of *A. arenosa* of more than 100 μg/g dry weight and of Zn of more than 3,000 μg/g dry weight in its natural habitat, the authors did not characterize this as hyperaccumulation (Kucharski et al., 2005; Nadgórska-Socha et al., 2013). Moreover, little information is available on a comparison of TME (Zn and Cd) accumulation and tolerance in *A. halleri* and *A. arenosa* (Szopiński et al., 2019).

Chlorophyll *a* fluorescence is commonly used to evaluate the overall photosynthetic performance in plants. The kinetics of chlorophyll *a* fluorescence is a very informative tool that is used for the non-invasive analysis of the effects of different environmental stresses such as Cd toxicity on photosynthesis (Baker, 2008; Kalaji et al., 2012; Kalaji, Govindjee, Bosa, Kościelniak, & Żuk-Gołaszewska, 2011; Kalaji, Oukarroum, et al., 2014; Kalaji, Schansker, et al., 2014; Rungrat et al., 2016; Sitko et al., 2017; Strasser, Tsimili-Michael, & Srivastava, 2004). Moreover, measuring the chlorophyll, flavonol and anthocyanin content has become a popular method for quickly analyzing the plant physiological status both in the field and in laboratory conditions (Gonzalez-Mendoza et al., 2017; Sitko et al., 2017; Szopiński et al., 2019).

The aim of this study was to perform an in-depth comparison of *A. arenosa* and *A. halleri* that are growing on the same contaminated site in Southern Poland together with two non-metallicolous populations from geographically close sites. In order to get a better understanding of any differences in the local adaptation of these two species to Cd contamination, their mineral composition, pigment content indices and photosynthetic apparatus performance were analyzed *in situ* and in a hydroponic experiment.

2 | MATERIALS AND METHODS

2.1 | Description of the sites and plant material

Field investigations were performed on two metallicolous (M) and two NM populations of *A. halleri* and *A. arenosa*. The M populations of *A. halleri* and *A. arenosa* are located on the same metalliferous site in Piekary Śląskie and are referred to in this paper as H-M and A-M, respectively. The H-M population that was used in this research is the same as the population that was referred to as P in Sitko et al. (2017). The NM population of *A. halleri* is located on a non-metalliferous site in Niepołomice and in this paper, it is referred to as H-NM. The H-NM population is the same as the PL13 population that was used by Meyer et al. (2010). The NM population of *A. arenosa* is located on a non-metalliferous site near Klucze and in this paper, it is referred to as A-NM (Table S1). The non-metalliferous sites in Klucze and Niepołomice are located 50 and 110 km away from the metalliferous site in Piekary Śląskie. Pictures of both the plants from the investigated population and their sites are presented in Figure S1. Figure S2 presents the siliques of the H-M and A-M populations from the site in Piekary Śląskie. It should be stressed that both the H-M and A-M plants produce flowers and siliques with viable seeds (Figures S1 & S2). The approximate locations of the investigated sites in Poland are presented in Figure S3. At each site, the populations had a large number of specimens and the sampling that was performed did not cause any significant injury to their diversity or abundance.

2.2 | Plant and soil samples collection and analysis

Eight individual plants from each population that were selected to measure their fluorescence and pigment content were collected along with a lump of soil in order to protect the root systems. In the laboratory, the leaves were separated from the roots and prewashed thoroughly in tap water and then washed using an ultrasound washer filled with deionized water. Subsequently, the plant samples were dried at 80°C for 72 hrs. Then, the dry plant material was ground in a mortar, mixed and acid-digested.

For each plant, three samples of the soil from its immediate surroundings were collected and mixed together in order to obtain one composite sample per plant. In the laboratory, the soil samples were air-dried and sieved through a 2 mm screen, and then used to measure the pH and EC and to analyze the mineral composition. Soil pH was measured in deionized water (1:2.5, m/vol) and 1 M KCl (1:2.5, m/vol) using a combination glass/calomel electrode (OSH 10–10; Metron, Poland) and a pH/conductivity metre (CPC-505; Elmetron, Poland) at room temperature after 24 hr of equilibration. The electrical conductivity was determined in a deionized water suspension (soil: solution ratio of 1:2.5, m/vol) at room temperature after 24 hr of equilibration using a glass conductivity cell (EC-60; Elmetron, Poland) and a pH/conductivity metre (CPC-505; Elmetron, Poland). The pseudo-total metal concentration was determined after the acid digestion of the soil, which had been ground to <0.25 mm. The concentration of the phytoavailable forms of the trace metal elements in the soil samples (Menzies, Donn, & Kopittke, 2007; Pueyo, López-Sánchez, & Rauret, 2004; Vondráčková, Hejcman, Száková, Müllerová, & Tlustoš, 2014) was assessed by extracting 3 g of dry soil that was then sifted to pass through a 2 mm sieve with 30 ml of 0.01 M $CaCl_2$ for 2 hr at room temperature (Sitko et al., 2017). The plant and soil material was digested in a microwave-assisted wet digestion system (ETHOS 1, Milestone, Italy) according to the procedure provided by the manufacturer (concentrated HNO_3 and H_2O_2, 4:1 vol/vol). The concentration of metals was analyzed in the extracts (soil, $CaCl_2$) and digests (plant, soil) using flame atomic absorption spectrophotometry (iCE 3,500 FAAS, Thermo Scientific). A reference plant (Oriental Basma Tobacco Leaves [INCT-OBTL-5], Institute of Nuclear Chemistry and Technology, Poland) and soil material (NCS DC 77302, China 502 National Analysis Center for Iron and Steel, Beijing, China) were used to determine the quality assurance of the analytical data.

The translocation factor (TF) for Cd and Zn was calculated as the TME concentration in the leaves of a plant divided by the TME concentration in the roots (Zhou et al., 2016).

The bioconcentration factor (BF) was calculated as the ratio between the TME concentration in the leaves and its concentration in the soil (Zhou et al., 2016).

2.3 | DNA content and ploidy level analysis

Young leaves from the flower stems of 15 individuals from the H-M, H-NM, A-M and A-NM populations were collected in the field. *Solanum lycopersicum L.* (formerly *Lycopersicon esculentum* cv. "Stupické polní tyčkové rané") (2C DNA content = 1.96 pg, Doležel, Sgorbati, & Lucretti, 1992) was used as the internal standard. 0.1 g of leaf material was mechanically fragmented in 400 µl of a Nuclei Extraction Buffer (CyStain® UV Precise P, 05–5,002, Sysmex) and the suspension of the nuclei was filtered through a 30-µm nylon mesh to remove any large debris and then stained with a Staining Buffer containing DAPI and 1% ß-mercaptoethanol (Sysmex) in order to prevent the samples from oxidizing. The samples were incubated for 1–2 min and analyzed using a CyFlow Space flow cytometer (Sysmex, Kobe, Japan) with a 365-nm UV LED diode as the light source. The ploidy level was estimated based on the fluorescence intensity by calculating the approximate DNA content of the individuals from each population. For the H-M and H-NM populations, the result for the 2C DNA content was approx. 0.49 and 0.48 pg, respectively (Table S1). The results that were obtained are very similar to previous reports, where the DNA content for the diploid *A. halleri* was estimated at approx. 0.45 pg (2C, where 1 pg = 978 Mbp) (Akama, Shimizu-Inatsugi, Shimizu, & Sese, 2014), ≈ 0.51 pg (2C, where 1 pg = 978 Mbp) (Briskine et al., 2017) and 0.52 pg (2C) (Johnston et al., 2005). The observed variances may have been because different ecotypes of *A. halleri* and different measurement methods were used. A similar computational principle was used for the A-M *and* A-NM populations. The calculated DNA content for this species was 0.808 pg (2C) for the A-NM population and 0.804 pg (2C) for the A-M population. These results are

similar to the DNA content that was estimated by Johnston et al. (2005) for the tetraploid *A. arenosa* (2n = 4x = 32, 2C = 0.834 pg), which indicates that the analyzed populations of *A. arenosa* are tetraploids (Table S1).

2.4 | Hydroponic cultures

The seeds of the plants from all four populations that were collected during the field investigations were sown in vermiculite that had been watered with deionized H_2O. After 2 weeks, the seedlings were watered with a 1/10 strength Hoagland solution for the next 2 weeks. After 4 weeks of growth on vermiculite, 24 individual seedlings from each population of *A. halleri* and *A. arenosa* were carefully transferred into hydroponic cultures in 16 plastic containers (6 seedlings/container). The containers were filled with 2.0 L of nutrient solution (333 ml/seedling). A ½ Hoagland solution (Bloom, 2006) was used in the hydroponic cultures, which had an initial pH of 5.8 ± 0.05. The seedlings were grown in a greenhouse under artificial light from sodium lamps (HPS), photoperiod 12:12 hr day/night, average light energy of 150 μmol E m^{-2} s^{-1}. The temperature in the greenhouse was 20 ± 1°C and the air humidity was 60% ± 5%. During the first 7 days of the experiment, the medium was not changed. After 7 days of plant cultivation, the medium was changed twice a week. The plants were grown for 5 weeks in the control solution. After 5 weeks, all of the containers were equally divided into experimental groups. Each group consisted of four containers with six individual plants from each population: a control group and three different treatments of Cd (50, 100 and 200 μM). The treatment lasted for 24 days and during this period, the medium was also changed twice a week. At the end of the experiment, all of the plants were collected and processed in the same way as was described for the field investigation.

2.5 | Chlorophyll *a* fluorescence and pigment content indices measurements

Fully developed leaves from the *A. halleri* and *A. arenosa* leaf-rosettes, which completely filled the area of the sensor, were selected for the measurements. Eight plants were selected from each population growing in the field. For each selected plant, four leaves were measured, always on the apical lobe of the leaf. Chlorophyll *a* fluorescence was measured using a Plant Efficiency Analyzer (PocketPEA fluorimeter, Hansatech Instruments Ltd., England). Before they were measured, each selected leaf was adapted in the dark for 30 min using leaf clips. After adaptation, a saturating light pulse of 3,500 μE m^{-2} s^{-1} was applied for 1 s, which closed all of the reaction centers and then the fluorescence parameters were measured. Fluorescence was measured *in situ* on interveinal area of leaves without destroying the plant material so that the clips did not overlap the midrib or the larger lateral vein.

The chlorophyll, flavonol and anthocyanin content indices *in situ* were measured on the same eight plants from each population as that were measured for the chlorophyll *a* fluorescence. The measurements were taken using a Dualex Scientific+ sensor (Force-A, France) without damaging the leaves.

In the laboratory experiment, at the end of Cd treatment, the chlorophyll *a* fluorescence was measured using the same method as those that were used in the field. The chlorophyll, flavonol and anthocyanin content indices were measured twice a week during the Cd treatment using a Dualex Scientific+ sensor (Force-A, France). Both the chlorophyll *a* fluorescence and pigment content were measured without destroying the plant material.

2.6 | Statistical analysis

The results are shown as the $M \pm SE$. The statistically significant differences among the mean values were determined using a one-way ANOVA and a post hoc Tukey HSD test ($p < .05$). The statistical analysis was performed using Statistica v.13.1 software (Dell Inc.). The pipeline models of the energy fluxes through a leaf's cross-section were created using CorelDRAW X6 (Corel Corp., Canada). The heat maps and hierarchical clustering analyses of the data about the mineral elements were performed using the "heatmap.2" function (GPLOTS R package). The values used to create the heat maps were calculated as $\log_2$ leaves: root ratio of the concentration of the mineral elements for each population and treatment (three biological replicates).

3 | RESULTS

3.1 | *A. arenosa* and *A. halleri* metal accumulation on their native sites

A field investigation was conducted in order to compare the metallicolous (M) and NM populations of *A. arenosa* and *A. halleri* growing on their native sites.

Plants from the M populations of *Arabidopsis halleri* (H-M) and *A. arenosa* (A-M) that were growing on the same site in Piekary Śląskie (south of Poland) were analyzed. The H-M grew on spots that had higher concentrations of the total and phytoavailable forms of TME compared to the A-M (Table 1). However, the concentrations of Cd and Zn in the H-M and A-M leaves were not statistically different (Figure 1a,b). The leaves: roots ratios were 2:8 and 3:0 for Cd and 4:3 and 3:2 for Zn in the A-M and H-M plants, respectively, which are typical for hyperaccumulators (Baker et al., 2000; Pollard et al., 2014; Van der Ent et al., 2013). The A-M plants were also characterized by a significantly higher accumulation of Fe and Mn in their leaves compared to the H-M plants (Figure 1c,d). On the other hand, there was no clear trend in the accumulation of Ca, Cu and Mg in the roots and leaves among the populations (Figure 1e–g).

The NM populations of *Arabidopsis halleri* from Niepołomice (H-NM) and the *A. arenosa* from Klucze (A-NM) were also analyzed. Despite the low Cd and Zn concentrations in both the Niepołomice

TABLE 1 Physicochemical properties of soils

Species	*A. arenosa*		*A. halleri*	
Edaphic type and names of the populations				
Name of the population Edaphic type	A-NM Non-metalliferous	A-M Metalliferous	H-NM Non-metalliferous	H-M Metalliferous
Physical properties of the soil				
pH (H_2O)	5.58 ± 0.10 b	6.70 ± 0.00 a	5.20 ± 0.00 c	6.74 ± 0.00 a
pH (KCl)	4.80 ± 0.12 b	6.57 ± 0.01 a	4.23 ± 0.06 c	6.48 ± 0.03 a
EC (μS/cm)	54.3 ± 9.89 b	124 ± 7.32 a	91.1 ± 13.8 a	164 ± 17.2 b
Total concentration of the elements in the soil (μg/g)				
Ca	3,070 ± 1,320 bc	6,550 ± 1,050 ab	1,620 ± 69.6 c	9,530 ± 1,560 a
Cd	8.44 ± 0.80 c	572 ± 60.1 b	2.46 ± 0.13 c	758 ± 59.4 a
Cu	19.3 ± 2.86 c	81.7 ± 10.5 b	15.6 ± 0.50 c	125.6 ± 15.1 a
Fe	8,780 ± 1,070 c	16,960 ± 1,170 ab	13,780 ± 520 b	20,630 ± 1,370 a
Mg	1,320 ± 505 a	1,660 ± 157 a	1,730 ± 103 a	2,240 ± 208 a
Mn	276 ± 35.1 c	755 ± 29.3 a	523 ± 27.8 b	875 ± 44.1 a
Pb	225 ± 24.4 c	9,070 ± 734 b	22.0 ± 2.18 d	11,100 ± 544 a
Zn	607 ± 97.6 b	18,980 ± 2,520 a	116 ± 7.15 c	27,720 ± 3,600 a
Concentration of the phytoavailable forms of TM in the soil (μg/g)				
Cd	0.52 ± 0.11 c	49.5 ± 3.02 b	0.18 ± 0.02 c	59.1 ± 1.06 a
Pb	0.20 ± 0.05 c	3.53 ± 0.73 b	0.04 ± 0.02 c	6.04 ± 0.93 a
Zn	20.2 ± 4.67 c	409 ± 14.6 b	6.22 ± 0.74 c	463 ± 12.8 a

Note: M—metallicolous; NM—non-metallicolous; the presented data are the means ± SE (n = 8). Means followed by the same letter in a row are not significantly different from each other according to the HSD test ($p \leq 0.05$). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous).

and Klucze soils (Table 1), the H-NM hyperaccumulated Cd (153 mg/kg DW) and it was close to the Zn hyperaccumulation threshold (2,790 mg/kg DW of the leaves), while the A-NM accumulated low concentrations of both metals (Figure 1a,b). Moreover, the H-NM plants had the highest concentration of Fe in the leaves (302 μg/g) compared to the other investigated populations (Figure 1c).

The A-NM, A-M and H-M populations growing on their native site showed similar leaves: soil BF for both Cd (from 17 to 22) and Zn (from 35 to 50), whereas the H-NM had an extraordinarily high BF of 963 and 539 for Cd and Zn, respectively (Table S2). The plants from the non-metallicolous H-NM and metallicolous A-M and H-M populations had a leaves: root TF for Cd that was more than one (Figure S4). In contrast, the A-NM population had a lower TF for Cd (leaves: root) compared to the other investigated populations. Interestingly, the Zn TF was higher for both of the *A. halleri* compared to the *A. arenosa* populations. By contrast, Fe was translocated from the roots to the leaves in both *A. arenosa* populations more effectively than in the *A. halleri* populations. On the non-metalliferous soil, the A-NM plants translocated more Zn than the H-NM plants, whereas the opposite trend was found on the metallicolous soil. Our results highlighted the different accumulation of Cd and Zn between *A. halleri* and *A. arenosa* in the field.

Besides Cd and Zn, the total concentration of Pb was measured in the metalliferous soil at the Piekary Śląskie site and ranged from 9,000 to 11,000 mg/kg. However, the concentration of the phytoavailable forms of Pb was low and did not exceed 6 mg/kg (Table 1) and it was 10-14-fold lower compared to Cd and 77-116-fold lower compared to the phytoavailable forms of Zn. In contrast to Cd and Zn, Pb accumulated predominantly in the plant roots (Figure 1h). As a result, the TF for Pb was low and ranged from 0.18 for the H-M plants to 0.28 for the A-M plants and it was even lower for the NM populations (TF from 0.10 to 0.18).

3.2 | Photosynthetic apparatus performance and pigment content analyses of the plants growing *in situ*

The chlorophyll *a* fluorescence changes are presented as the typical OJIP transient on a logarithmic scale because the fluorescence was measured at the 1 s (Figure 2a). Changes in the kinetics of the OJIP fluorescence were determined by calculating the differences in the variable fluorescence curves ΔV_t, where $V_t = (F_t - F_0)/(F_m - F_0)$, V_t represents the variable fluorescence, F_0 represents the minimal fluorescence and F_m represents the maximal fluorescence (Figure 2b). ΔV_t was calculated as the difference between the mean values of the fluorescence for the M population (A-M and H-M) and the mean values of the fluorescence for the NM plants from the same species (A-NM and H-NM). Hence, the fluorescence of the plants from the non-metallicolous populations (A-NM for *A. arenosa* and H-NM for

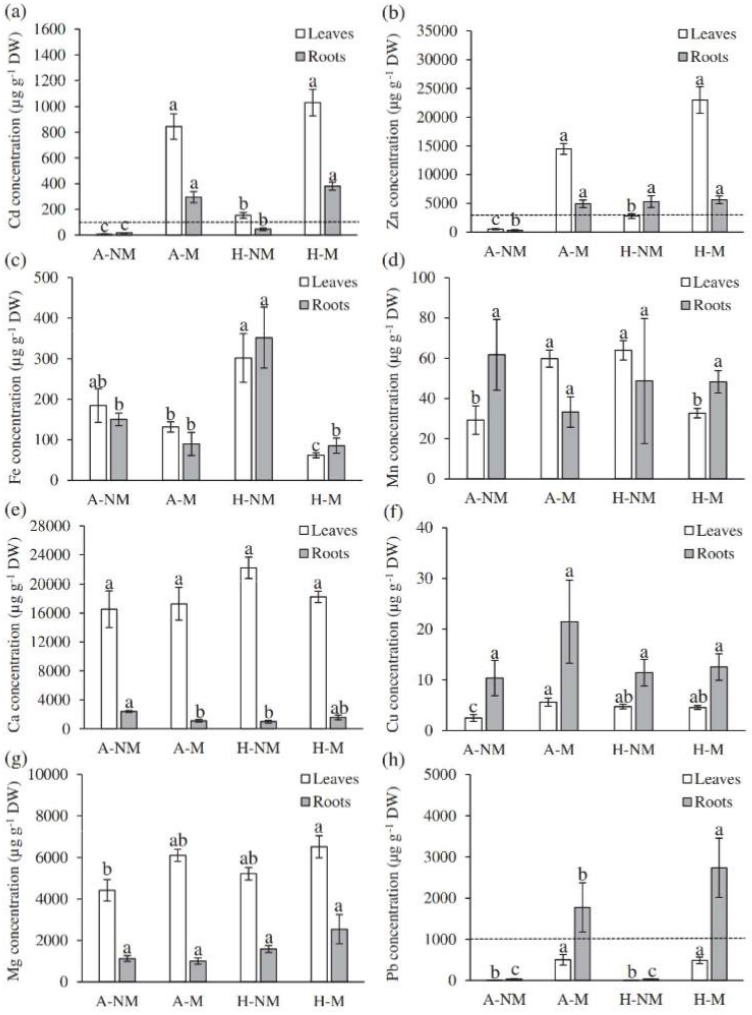

FIGURE 1 Accumulation of Cd (a), Zn (b), Fe (c), Mn (d), Ca (e), Cu (f), Mg (g) and Pb (h) in the leaves and roots of the plants from the metallicolous (M) and non-metallicolous (NM) populations of *A. arenosa* and *A. halleri in situ*. The dotted line represents the hyperaccumulation threshold. Data are the means ± SE (n = 8). Means followed by the same letter are not significantly different from each other according to the HSD test ($p \leq .05$). Statistical analysis was done separately for the leaves and roots. Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous)

A. halleri) was zero and was used as the reference. The theoretical base and parameters that describe photosynthesis performance are summarized in Table S3.

Both of the M populations were characterized by slightly higher values in the ΔK point compared to the NM populations, which indicates that the oxygen evolving complex (OEC) was less active than in their non-metallicolous reference populations. There was a distinct peak for step ΔJ for the A-M population, which had a higher value than in the H-M, however, both metallicolous populations had a lower reoxidation rate of Q_A^- than their respective NM populations (Figure 2b). The negative values in step ΔH and ΔG, which were observed for both M populations, suggests a higher activity of the PSI end electron acceptors such as Ferredoxin-$NADP^+$ Reductase (FNR) compared to the NM populations (Figure 2b). Thus, we found that in the field both NM populations were characterized by a higher efficiency of the photosynthetic apparatus with the exception of the end elements of the electron transport chain such as FNR, which seemed to be less affected in the M populations.

Both *A. arenosa* populations had a similar efficiency of their photosystems. The H-NM plants had the highest percentage of active RC (100%), absorption flux (ABS), trapped energy flux (TR) and electron transport flux (ET) among all of the investigated populations (Figure S5). By contrast, the H-M population was characterized by the highest percentage of inactive RC (39%), however, this difference was not significant compared to the A-M and A-NM populations. The plants from the H-M population also had the lowest intensity of

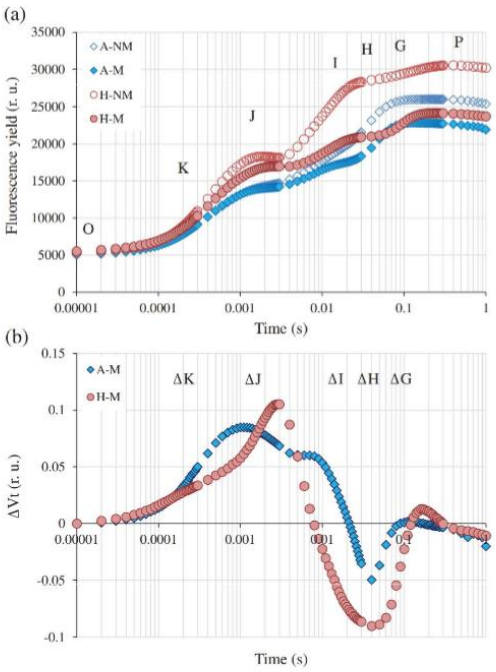

FIGURE 2 The chlorophyll *a* fluorescence induction curve of the *A. halleri* and *A. arenosa* plants from the metalliferous (M) and non-metalliferous (NM) sites (a) and the relative variable fluorescence ($\Delta V_t = ((F_t - F_0)/F_v) - V_{tF}$) of the plants from the metallicolous populations (b). For the ΔV_t analysis, the fluorescence of the plants from the non-metallicolous populations (A-NM for A-M and H-NM for H-M) was the reference and equalled 0. The values are the means (n = 25). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous) (open diamonds); A-M—*A. arenosa* from Piekary Śląskie (metalliferous) (closed diamonds); H-NM—*A. halleri* from Niepołomice (non-metalliferous) (open circles); H-M—*A. halleri* from Piekary Śląskie (metalliferous) (closed circles); r.u., relative units

absorption, trapped energy and electron transport flux compared to the other populations (Figure S5).

The H-NM population had the highest value of the maximum quantum yield (φP_0), which reflects the physiological status of a plant (Table S4), whereas the A-NM, A-M and H-M populations had similar values of φP_0. The H-M population had the lowest values of ψE_0 and φE_0, which describe the yield of the electron transport and the longest time to reach the maximum fluorescence, respectively (Table S4).

Both the M and NM *A. arenosa* populations had a significantly higher chlorophyll content index compared to the *A. halleri* populations *in situ* (Figure 3a). A considerably lower chlorophyll content index was measured in the H-NM population compared to the H-M population. By contrast, there was no significant difference between the two *A. arenosa* populations (Figure 3a). Analysis of the flavonol and anthocyanin content indices in the leaves revealed that both metallicolous populations of each investigated species had a significantly higher content index of these pigments compared to their non-metallicolous counterparts (Figure 3b,c).

3.3 | Relationships among the soil properties, photosynthesis, pigment content and accumulation of metals in the leaves *in situ*

A principal component analysis (PCA) that was conducted on the ionomic data and photosynthesis parameters highlighted the edaphic type as the factor that had the strongest impact on the population distributions (Figure 4). The metallicolous populations were separated from the non-metallicolous by the first principal component (Factor 1), which accounted for 60.47% of the variance.

3.4 | Tolerance and metal accumulation of *A. halleri* and *A. arenosa* in controlled conditions

A hydroponic culture was used to compare *A. arenosa* and *A. halleri* in controlled conditions. The plants were treated with 50, 100 and 200 μM of Cd or grown without added Cd for 24 days.

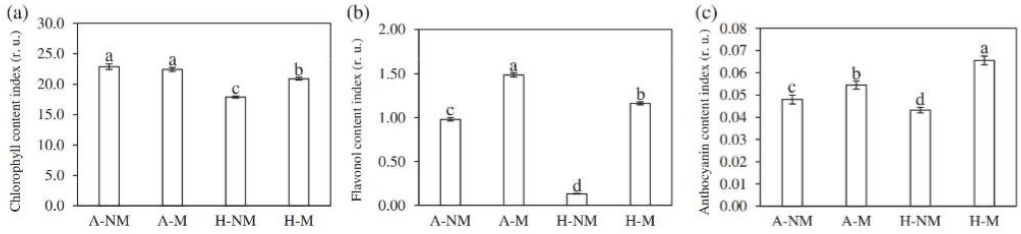

FIGURE 3 The chlorophyll (a), flavonol (b) and anthocyanin (c) content indices in the leaves of the plants from the metallicolous (M) and non-metallicolous (NM) populations of *A. arenosa* and *A. halleri* plants *in situ*. The values are the means ± SE (n = 20). Means followed by the same letter are not significantly different from each other according to the HSD test ($p \leq .05$). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous); r.u., relative units

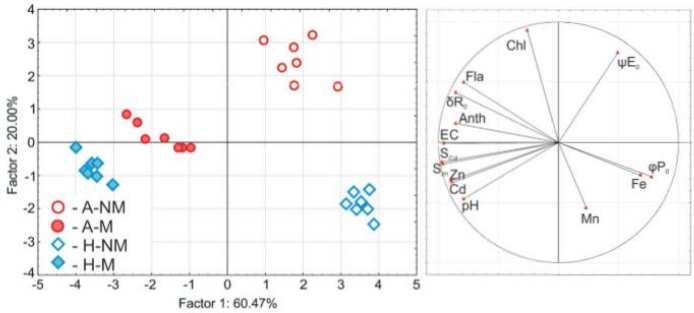

FIGURE 4 Principal component analysis (PCA) presenting the relationships between the soil properties, accumulation of TMs in the leaves, the pigment content indices and some of the photosynthetic parameters of the *Arabidopsis arenosa* and *Arabidopsis halleri* plants *in situ*. Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous). Parameters: Cd—accumulation of Cd in leaves; Fe—accumulation of Fe in leaves; Mn—accumulation of Mn in the leaves; Zn—accumulation of Zn in the leaves; Chl—chlorophyll content index; Fla—flavonol content index; Anth—anthocyanin content index; φP_0—maximum quantum yield of the primary PSII photochemistry; ψE_0—probability (at t = 0) that a trapped exciton will move an electron into the electron transport chain beyond Q_A; δR_0—probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side; EC—electrical conductivity of the soil; pH—pH(H_2O) of the soil; S_{Cd}—concentration of the phytoavailable forms of Cd in the soil; S_{Zn}—concentration of phytoavailable forms of Zn in the soil

Along with the increase in the Cd concentration in the medium, the biomass of the leaves and roots decreased in all of the investigated populations. *A. arenosa* generally had a higher biomass than *A. halleri* in the control conditions and the metallicolous populations were generally more tolerant to the Cd treatments than the non-metallicolous populations (Figure 5a,b). The biomass of the leaves in both of the M populations under 200 μM of Cd decreased by 70% compared to the control, a similar trend was observed in the NM populations with an appr. 80% decrease in the A-NM and H-NM (Figure 5a). In the A-M and H-M plants that had been treated with 200 μM of Cd, the biomass of the roots was similarly affected and decreased by less than 50%. On the other hand, the same concentration of Cd caused a 90 and 84% root growth inhibition in the A-NM and H-NM plants compared to the untreated plants, respectively (Figure 5b).

The H-NM population accumulated more Cd and Zn in the leaves compared to the other populations (Table 2). There was also a higher Zn accumulation in the leaves of the H-M population compared to both the A-NM and A-M populations. In general, the concentrations of Cu, Fe and Mn decreased along with an increase of the Cd concentration in the leaves (Table 2).

The accumulation of Cd in the roots was higher in *A. arenosa* compared to the *A. halleri* populations (Table S5). Cd treatment caused a large decrease in the Mn concentration in the roots of all of the investigated populations, while it had no significant effect on the Zn accumulation in any of the populations (Table S5).

In the H-M population, the root-to-shoot translocation of Cd was lower compared to the H-NM population (Figure S6), whereas the opposite trend was found in the *A. arenosa* populations. Both the M populations translocated more Mn into the leaves than their NM counterparts. There was also a higher TF for Zn in the *A. halleri* populations compared to the *A. arenosa* populations (Figure S6).

3.5 | Photosynthetic apparatus performance and pigment content of *A. halleri* and *A. arenosa* in controlled conditions

The ΔV_t for the plants that had been treated with 200 μM of Cd (Figure 6b) was calculated as the difference between the mean values of the fluorescence for each population that had been treated with 200 μM of Cd (A-NM 200, A-M 200, H-NM 200 and H-M 200) and the mean values of the fluorescence of the plants from the control for each population. Hence, the fluorescence of the plants from control group was zero and was used as the reference.

The photosynthetic apparatus of both of the metallicolous populations had a higher tolerance to Cd compared to the non-metallicolous populations and the H-M population was the most tolerant (Figure 6a). Moreover, an analysis of the relative variable fluorescence showed that the elements of the photosynthetic apparatus in the H-M population were not significantly damaged by 200 μM of Cd (no ΔI, ΔH, and ΔG peaks) compared to the control (Figure 6b). The A-M plants that had been treated with 200 μM of Cd had increased values in the ΔK, ΔJ, and ΔI points, which suggest a toxic effect of Cd. However, the highest values in the ΔK, ΔJ, and ΔI points were observed in the A-NM population (Figure 6b).

In both non-metallicolous populations, Cd drastically reduced the percentage of active reaction centers (RC). By contrast, the H-M population was much more resistant and even after being treated with 200 μM of Cd, there was only a moderate decline in the physiological

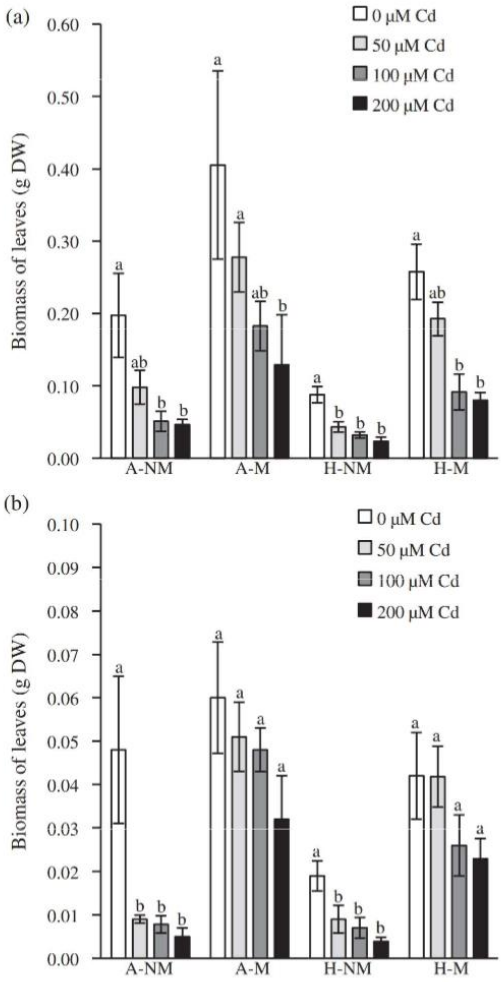

FIGURE 5 Toxic effect of Cd on the growth of the leaves (a) and roots (b) of the plants from the metallicolous (M) and non-metallicolous (NM) populations of *A. arenosa* and *A. halleri* plants after 24 days of Cd treatment. The values are the means ± SE ($n = 6$). Means followed by the same letter are not significantly different from each other within a population according to the HSD test ($p \leq .05$). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous)

status of PSII (Figure S7). In the A-M population, most of the parameters were severely affected when the plants were treated with 100 μM of Cd (Figure S7). Nevertheless, the photosynthetic apparatus of the A-M population was significantly more resistant to Cd than either of the NM populations (A-NM, H-NM). In the A-NM and H-NM plants, even 50 μM of Cd in the growth medium considerably lowered all of the analyzed parameters of the PSII (Figure S7).

The maximum quantum yield (φP_0) was similar in all of the populations in the control medium. φP_0 was lowered by all of the Cd concentrations in the plants from the A-NM and H-NM populations (Table S6). By contrast, only the highest concentration of Cd caused a substantial decrease in φP_0 for the A-M populations compared to the control. On the other hand, in the H-M plants, the φP_0 values were not affected by even the highest Cd treatment. Moreover, the H-M population was the only population whose yield of electron transport (ψE_0) was not affected by any of the Cd treatments (Table S6).

After 24 days of Cd treatment, the chlorophyll content index visibly decreased in both of the NM populations independently of the Cd concentration in the medium, whereas in both of the M populations, there was only a gradual decrease compared to the control with an increase in the Cd concentration (Figure 7a).

In the control conditions, the anthocyanin content index was similar among all of the investigated populations, while the flavonol content index was significantly higher in both the M populations (Figure 7b,c). The flavonol content index increased after the Cd treatment in the *A. arenosa* populations, especially in the A-NM, whereas it was unaffected in the *A. halleri* populations (Figure 7b). There was a strong increase in the anthocyanin content index after Cd treatment in both of the NM populations, irrespective of the Cd concentration. On the other hand, the increase in the content of anthocyanins after Cd treatment in the M populations was less pronounced compared to the NM plants (Figure 7c). The changes in chlorophyll, flavonol and anthocyanin content indices during the 24-day treatment with 50, 100 and 200 μM of Cd are presented in the supplementary material (Figures S8–S10).

3.6 | Relationships among the metal accumulation in leaves, photosynthesis and pigment content analyses of *A. halleri* and *A. arenosa* populations in the controlled conditions

PCA analysis on the ionomic data, photosynthesis and pigment content indices of the plants growing in hydroponic conditions showed that in the control conditions, both the *A. arenosa* populations had similar features, which grouped them together (Figure 8). By contrast, the H-M population was grouped separately from the H-NM population because of the differences in the Zn accumulation in the leaves, the efficiency of the PSII and chlorophyll content index. In general, the Cd treatments distinctively separated the NM and M populations. In this new trend, the accumulation of Cd, anthocyanin and chlorophyll content indices had the greatest impact on the explained variance, and there was a negative correlation between the chlorophyll and anthocyanin content indices. The separate grouping of the NM and M populations in the PCA analysis further supports differences in the tolerance in the M and NM plants (Figure 8).

TABLE 2 Accumulation of the elements in the leaves of *A. arenosa* and *A. halleri* after different Cd treatments (μg/g DW)

Species		*A. arenosa*		*A. halleri*	
Name of the population		A-NM	A-M	H-NM	H-M
Accumulation of elements in leaves					
Ca	0 μM Cd	44,700 ± 1,860 aAB	30,770 ± 3,310 cA	39,820 ± 1,700 abB	30,900 ± 1,340 bcAB
	50 μM Cd	49,920 ± 2,070 aA	32,200 ± 2,000 bA	47,100 ± 3,310 aAB	26,530 ± 1,550 bB
	100 μM Cd	54,760 ± 3,180 aA	36,430 ± 1,780 bA	52,640 ± 2,340 aA	33,040 ± 890 bA
	200 μM Cd	34,620 ± 2,500 bB	36,800 ± 2,790 bA	48,120 ± 2,460 aAB	32,540 ± 1,850 bA
Cd	0 μM Cd	4.69 ± 0.75 bC	6.23 ± 0.73 bD	6.56 ± 0.56 bD	10.4 ± 0.89 aD
	50 μM Cd	2,330 ± 236 bB	1,770 ± 452 bC	3,280 ± 222 aC	1,750 ± 141 bC
	100 μM Cd	4,810 ± 345 abA	4,120 ± 276 bB	5,830 ± 491 aB	4,450 ± 171 abB
	200 μM Cd	4,170 ± 598 bA	7,140 ± 639 aA	8,320 ± 761 aA	7,230 ± 564 aA
Cu	0 μM Cd	32.4 ± 2.94 aA	33.0 ± 3.77 aA	38.4 ± 3.99 aA	11.0 ± 1.08 bA
	50 μM Cd	21.5 ± 4.82 aAB	20.0 ± 2.24 aB	12.5 ± 1.33 abB	7.94 ± 0.97 bAB
	100 μM Cd	11.4 ± 1.88 abBC	19.6 ± 3.42 aB	11.0 ± 1.64 abB	5.58 ± 0.88 bB
	200 μM Cd	5.56 ± 1.08 aC	9.81 ± 2.68 aB	7.53 ± 0.97 aB	9.16 ± 1.13 aAB
Fe	0 μM Cd	39.6 ± 7.20 bA	42.1 ± 2.87 abA	44.4 ± 3.57 abA	63.4 ± 6.61 aA
	50 μM Cd	16.9 ± 2.80 aB	22.1 ± 3.00 aB	28.2 ± 4.14 aB	28.5 ± 3.68 aB
	100 μM Cd	7.28 ± 1.14 aB	8.57 ± 0.81 aC	16.6 ± 4.74 aB	21.2 ± 5.22 aB
	200 μM Cd	5.58 ± 0.61 abB	7.54 ± 0.86 abC	4.51 ± 1 bC	11.4 ± 2.49 aB
Mg	0 μM Cd	6,260 ± 398 aB	4,020 ± 398 bB	6,210 ± 564 aB	4,940 ± 158 abB
	50 μM Cd	8,540 ± 349 aA	6,640 ± 410 bB	8,030 ± 456 abAB	4,520 ± 202 cB
	100 μM Cd	8,850 ± 454 abA	6,350 ± 616 bcB	8,920 ± 924 aA	6,260 ± 156 cA
	200 μM Cd	5,600 ± 483 bB	7,090 ± 493 abA	7,770 ± 517 aAB	6,610 ± 373 abA
Mn	0 μM Cd	121 ± 13.5 bA	220 ± 26.7 aA	245 ± 21.2 aA	279 ± 12.2 aA
	50 μM Cd	116 ± 4.87 aA	119 ± 7.02 aB	158 ± 12.3 aB	139 ± 15.8 aB
	100 μM Cd	124 ± 7.45 abA	97.9 ± 5.51 bB	164 ± 13.9 aB	161 ± 16.7 aB
	200 μM Cd	93 ± 5.95 bA	113 ± 8.93 bB	161 ± 15.7 aB	116 ± 7.77 bB
Zn	0 μM Cd	110 ± 17.1 cA	150 ± 17.9 cA	1,480 ± 147 aA	406 ± 33.0 bA
	50 μM Cd	106 ± 11.4 cA	90.5 ± 5.75 cB	1,450 ± 176 aA	258 ± 39.6 bA
	100 μM Cd	100 ± 12.3 cA	91.5 ± 7.24 cB	1,210 ± 103 aA	334 ± 63.1 bA
	200 μM Cd	109 ± 48.0 cA	81.1 ± 10.9 cB	966 ± 124 aA	253 ± 31.2 bA

Note: M—metallicolous; NM—non-metallicolous; the presented data are the means ± SE ($n = 6$). Means followed by the same lower case letter in a row and an upper case letter in a column (for a single element) are not significantly different from each other according to the HSD test ($p \leq .05$). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous).

4 | DISCUSSION

4.1 | *Arabidopsis halleri* and *A. arenosa* had contrasting metal accumulation strategies

Arabidopsis arenosa is a facultative pseudometallophyte (Bothe & Słomka, 2017; Wójcik et al., 2017). However, the mechanisms that have evolved in *A. arenosa* to cope with an excess of metals are poorly known and there is little information concerning its metal accumulation capacity and tolerance *in situ* or in controlled conditions (Bothe & Słomka, 2017; Preite et al., 2019; Przedpełska-Wąsowicz & Wąsowicz, 2013; Rozpądek et al., 2018; Staňová et al., 2012; Szarek-Łukaszewska & Grodzińska, 2007; Szopiński et al., 2019; Wójcik et al., 2017).

Our field investigations showed that on the metalliferous site in Piekary Śląskie (Table 1), the *A. arenosa* population (A-M) accumulates Cd and Zn in the leaves at similar concentrations as the known hyperaccumulator *A. halleri* (H-M) on the same site (Figure 1a,b). These results are consistent with the data that was previously published for *A. halleri* and *Noccaea caerulescens* growing in contaminated soils (Galiová et al., 2019; Sitko et al., 2017; Stein et al., 2017). Kucharski et al. (2005) and Nadgórska-Socha et al. (2013) showed that the *A. arenosa* populations from Southern Poland are capable of accumulating Cd and Zn in their leaves at a level that exceeds the hyperaccumulation threshold. These observations, however, were not linked to the hyperaccumulation phenomenon. A hierarchical cluster analysis based on the leaves: root ratio of the mineral elements

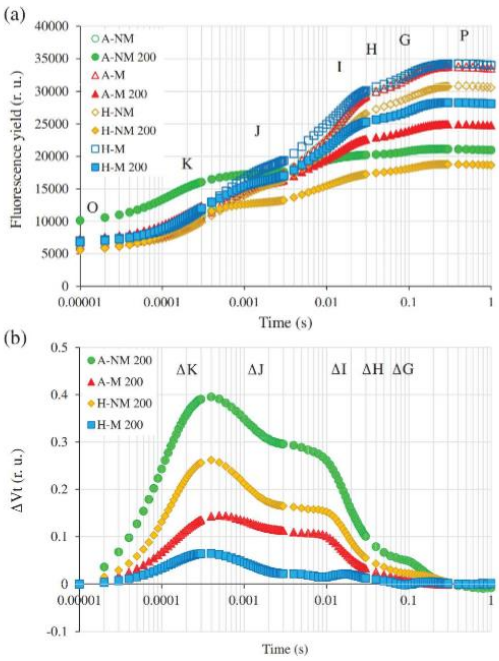

FIGURE 6 The chlorophyll *a* fluorescence induction curve of *A. halleri* and *A. arenosa* in the control medium and those that had been treated with Cd at a concentration of 200 μM (a) and the relative variable fluorescence ($\Delta V_t = ((F_t - F_0)/F_v) - V_{tF}$) of the Cd-treated plants (b). For the ΔVt analysis, the fluorescence of the control plants from each population (A-NM, A-M, H-NM, H-M) was regarded as the reference and equalled 0. The values are the means (n = 15). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous). r.u., relative units. 200—treatment with 200 μM of Cd

grouped the A-M population closer to both of the *A. halleri* hyperaccumulator populations (H-M, H-NM) than to its NM counterparts (A-NM) (Figure S4), thus further suggesting that the A-M population had metal accumulation strategies that are similar to the hyperaccumulators of Cd and Zn. However, in contrast to *A. halleri* in which the hyperaccumulation of Cd and Zn was observed in both the M and NM populations, the hyperaccumulation in *A. arenosa* was only observed in the M populations.

It is also noteworthy that *in situ* H-NM hyperaccumulated Cd and accumulated Zn close to the hyperaccumulation threshold despite the low level of these elements in the soil (Table 1). This result is in agreement with Stein et al. (2017), who showed that the Cd and Zn accumulations are highly variable among *A. halleri* populations and do not depend on the edaphic type. In the present study, the ratio of the H-NM population for hyperaccumulator:non-hyperaccumulator individuals was 7:1 for Cd and 4:4 for Zn, which suggest that there is a large polymorphism in this population.

Lead is a metal that easily precipitates in the presence of phosphate ions. This precipitation is also observed when the pH of a solution rises above 6.0. As a result, in a large number of the experiments that have been conducted with Pb in the presence of phosphate ions or at pH above 6.0, the toxicity of Pb was relatively low, particularly compared to Cd (Małkowski, Sitko, Zieleźnik-Rusinowska, Gieroń, & Szopiński, 2019). However, it was recently proved that the toxicity of Pb to plants is very high when a hydroponic medium with a low pH and a low concentration of phosphate ions is used (Fisher, Kühnlenz, Thieme, Schmidt, & Clemens, 2014; Kopittke, Asher, & Menzies, 2008; Shahid, Pinelli, Pourrut, Silvestre, & Dumat, 2011). Based on these types of experiments, Kopittke, Blamey, Asher, and Menzies (2010) proposed that Pb is the most phytotoxic metal compared to the TMEs such as Cu, Cd, Ni and Zn.

The metalliferous soil in Piekary Śląskie was not only highly contaminated with Cd and Zn, but also with Pb (Table 1), which is typical for soils in the vicinity of Pb/Zn smelters (Kapusta, Szarek-Łukaszewska, & Stefanowicz, 2011; Kucharski et al., 2005; Wójcik, Sugier, & Siebielec, 2014). Although the total concentration of Pb in the soil was high and reached as much as 11,000 mg/kg, the concentration of the phytoavailable forms was low compared to Cd or Zn (Table 1). Such a low availability of this metal in soils that have been contaminated by nonferrous smelters or mines has also been documented by different authors (e.g., Gucwa-Przepióra et al., 2007; Kapusta et al., 2011; Wójcik et al., 2014). As a result, relatively low concentrations of Pb were found in plants on the metalliferous site, particularly in the leaves, because of the low TF for Pb (Figure 1h). Taking into account the high concentration of the phytoavailable forms of Cd (50–59 mg/kg) and Zn (409–460 mg/kg) compared to Pb (3.5–6.0 mg/kg), it is suggested that the main factors behind the adaptation of both of the investigated plant species to the toxicity of TMEs on the metalliferous site are Cd and Zn. This notion is supported by other authors, who also measured low concentrations of the bioavailable forms of Pb in metalliferous soils in Southern Poland and postulated that the effect of Pb on living organisms (soil mesofauna, microbes, arbuscular mycorrhizal fungi and plants) is negligible or substantially lower than that of Cd and Zn (Gucwa-Przepióra, Błaszkowski, Kurtyka, Małkowski, & Małkowski, 2013; Kapusta et al., 2011; Kapusta, Szarek-Łukaszewska, Jędrzejczyk-Korycińska, and Zagórna (2015). Moreover, it has been demonstrated that the Pb distribution in the *A. halleri* plants was different compared to Cd and Zn, which suggests the evolution of different accumulation pathways and strategies (Höreth et al., 2020). Therefore, we cannot exclude the fact that Pb affects plant growth and development on the metalliferous site in Piekary Śląskie and further research is necessary.

The plants growing in the hydroponic culture in the control and with different Cd concentrations enabled the metal accumulation dynamics in the investigated populations to be compared better. When exposed to the lowest Cd concentration, both the A-NM and H-NM populations accumulated more Cd in the leaves (Table 2) than

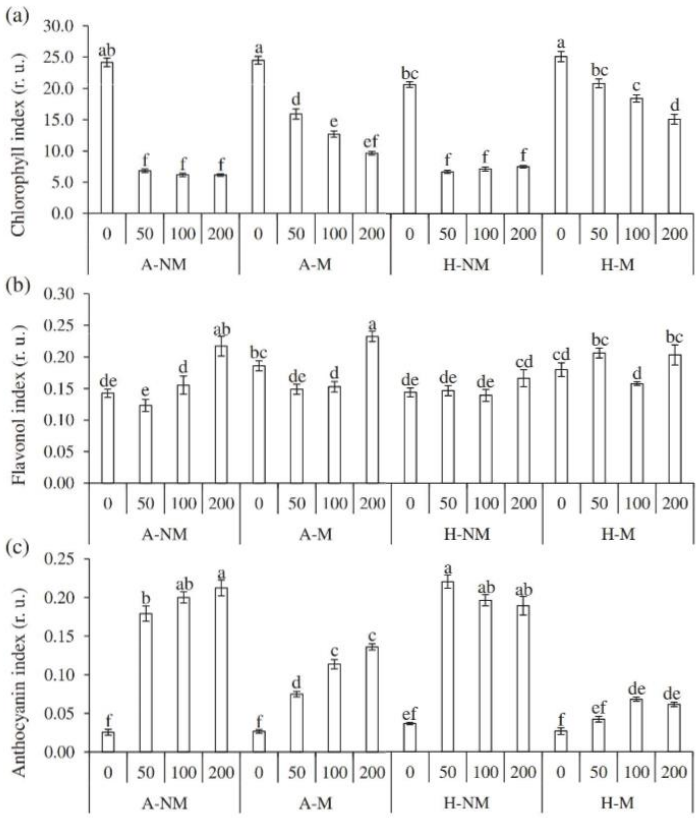

FIGURE 7 The chlorophyll (a), flavonol (b) and anthocyanin (c) content indices in the leaves of plants from the metallicolous (M) and non-metallicolous (NM) populations of *A. arenosa* and *A. halleri* after 24 days of 0, 50, 100 or 200 μM Cd treatment. The values are the means ± SE (n = 20). Means followed by the same letter are not significantly different from each other according to the HSD test ($p \leq .05$). Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous). r.u., relative units

the M populations, which suggests a local adaptation to contaminated sites by limiting the uptake of Cd.

The H-NM plants had the greatest capacity to hyperaccumulate Cd. When they were subjected to the highest Cd concentration in the hydroponic culture, they accumulated a twofold higher Cd than the A-NM, but the plants showed symptoms of high toxicity (Figures 5 & 6) and were damaged. On the other hand, the A-M plants accumulated similar Cd concentrations in the leaves as the hyperaccumulator H-M and had no symptoms of toxicity. The higher tolerance of the *A. halleri* M populations to Cd compared to the NM populations and non-hyperaccumulator species *A. lyrata* was already reported in controlled conditions (Meyer et al., 2015). We showed that Cd treatments negatively affected the Zn accumulation only in the A-M population, which may suggest different mechanisms of Cd and Zn transport in the A-M and H-M plants. Szopiński et al. (2019) provided strong support for the contrasting accumulation of Cd and Zn in hydroponic conditions between the same M populations of *A. halleri* and *A. arenosa* as were used in the current study. They showed that the A-M plants that had been subjected to very high Cd and Zn concentrations for 5 days accumulated three times more Cd than the H-M, while *A. halleri* accumulated more than threefold Zn compared to *A. arenosa* when exposed to extremely high concentrations of these TME. Furthermore, in the current study, H-NM and H-M populations also had a greater capacity to accumulate Zn than *A. arenosa*. Interestingly, the H-NM plants accumulated extremely high concentrations of Zn in the control conditions, which suggests highly efficient mechanisms for Zn accumulation in this population. Previous reports have shown competition in the uptake and translocation between Zn and Cd in *A. halleri* (Küpper, Lombi, Zhao, & McGrath, 2000; Przedpełska-Wąsowicz, Polatajko, & Wierzbicka, 2012). However, our results are in agreement with Huguet et al. (2012), who observed no change in Zn accumulation when the plants were exposed to Cd (20 μM) for 3 and 9 weeks. The competition between Cd and Zn might depend on the experimental conditions (e.g., exposure time and mineral composition of growth medium) and on the studied population (Huguet et al., 2012).

In general, the Cd treatments caused lower accumulation of Cu, Fe and Mn and an increase in the Mg and Ca accumulation (Table 2). A positive correlation between the Mg accumulation and Cd concentration has also been observed in *A. halleri* by other authors (Przedpełska-Wąsowicz et al., 2012; Sitko et al., 2017; Stein

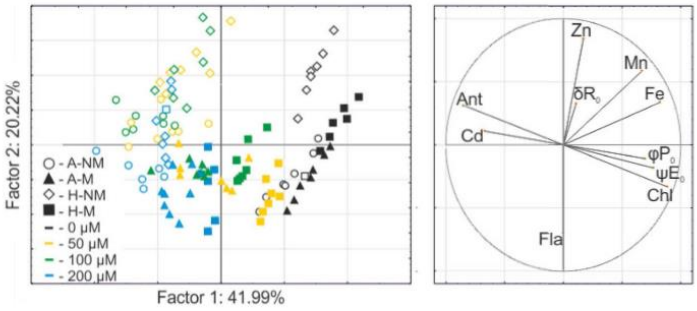

FIGURE 8 Principal component analysis (PCA) presenting the relationships between the TME accumulation in the leaves, the pigment content indices and some of the photosynthetic parameters of the *Arabidopsis arenosa* and *Arabidopsis halleri* plants that had been treated with Cd at a concentration of 0 (black), 50 (yellow), 100 (green) and 200 (blue) µM. Abbreviations of the plant population names: A-NM—*A. arenosa* from Klucze (non-metalliferous); A-M—*A. arenosa* from Piekary Śląskie (metalliferous); H-NM—*A. halleri* from Niepołomice (non-metalliferous); H-M—*A. halleri* from Piekary Śląskie (metalliferous). Parameters: Cd—accumulation of Cd in the leaves; Fe—accumulation of Fe in the leaves; Mn—accumulation of Mn in the leaves; Zn—accumulation of Zn in the leaves; Chl—chlorophyll content index; Fla—flavonol content index; Anth—anthocyanin content index; φP_0—the maximum quantum yield of the primary PSII photochemistry; ψE_0—probability (at t = 0) that a trapped exciton will move an electron into the electron transport chain beyond Q_A; δR_0—probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side

et al., 2017). The decreased Fe accumulation that was observed in our experiment might be because of Fe/Cd competition (Meyer et al., 2015). Indeed, the main Fe transporter in roots, IRT1, is non-specific and can also transport Cd (Kerkeb et al., 2008; Shanmugam, Lo, & Yeh, 2013; Vert et al., 2002). This competition was observed in all of the four studied populations. Moreover, the negative impact of Cd on the Mn content, which is documented in the current study, suggests that Cd competes with Mn for transport through the same non-specific transporters from the ZIP, NRAMP and/or MTP families (Cailliatte, Schikora, Briat, Mari, & Curie, 2010; Ishimaru et al., 2012; Küpper & Kochian, 2010; Mizuno et al., 2005; Pittman, 2005; Sasaki, Yamaji, Yokosho, & Ma, 2012; Zhang & Liu, 2017).

The results that were obtained from the hydroponic experiment confirmed the high ability of the A-M population to accumulate and tolerate a high concentration of Cd, which was similar to the H-M population (Figure 5a,b). By contrast, in both NM populations, the growth was drastically reduced by the Cd treatment as has been shown in other studies (e.g., Corso et al., 2018; Meyer et al., 2015; Schvartzman et al., 2018). Our results documented that the population of *A. arenosa* from Piekary Śląskie (A-M) developed Cd hypertolerance and hyperaccumulation traits, but that they, at least partially, rely on different mechanisms compared to *A. halleri*. It is also important to mention that the studied populations of *A. arenosa* and *A. halleri* have different ploidy levels (Table S1), which limits the genetic exchange between these two species. Preite et al. (2019) suggested that the *A. arenosa* and *A. halleri* populations from the same M sites underwent convergent evolution, which resulted in a parallel adaptation to a high concentration of TME, which additionally supports the results presented in the current study.

4.2 | Toxic effect of Cd on photosynthetic apparatus

The impact of Cd toxicity on the photosynthetic apparatus has been studied in several plant species (Gill, Khan, & Tuteja, 2012; Kalaji & Loboda, 2007; Paunov, Koleva, Vassilev, Vangronsveld, & Goltsev, 2018). However, there is a paucity of data on the impact of toxic TME on the photosynthetic apparatus in the metal hyperaccumulator and hypertolerant plant species *in situ* (Sitko et al., 2017) and in controlled conditions (Bayçu et al., 2017; Bayçu, Moustaka, Gevrek-Kürüm, & Moustakas, 2018; Küpper, Parameswaran, Leitenmaier, Trtílek, & Šetlík, 2007; Moustakas et al., 2019). Specifically, there is limited information for *A. arenosa* (Szopiński et al., 2019) and therefore we presented the photosynthetic status for this species both *in situ* and in laboratory conditions.

An analysis of the chlorophyll content can be used as an indicator of plant tolerance to different stress factors including toxic TME (Ramirez et al., 2014; Szopiński et al., 2019). Our results in the field showed significantly higher chlorophyll content index in both of the *A. arenosa* populations compared to the *A. halleri* populations (Figure 3a), which suggests that it might be a species-specific trait for *A. arenosa*. The higher chlorophyll content index in the A-M plants compared to the H-M plants from the same metalliferous site might be evidence of a better physiological status of this population. The very low chlorophyll content index in the leaves of the H-NM plants might be connected with the shade conditions in which this population grows in Niepołomice (non-metalliferous). Such a response of plants to shade conditions was also found by Zivcak, Brestic, Kalaji, and Govindjee (2014) and Sitko et al. (2017). As was previously shown (e.g., Meyer et al., 2015), we observed a higher tolerance to the toxic

effect of Cd in both the A-M and H-M populations compared to the NM populations (Figure 7a).

Despite growing on a site that was heavily contaminated with TME, the efficiency of the photosynthetic apparatus was high in both the A-M and H-M populations (Figure 2b). Taking into account the accumulation of Cd and Zn in the leaves in both M populations, the φP_0 value indicated that the PSII efficiency was not significantly affected (Table S4) and it was comparable to that of the M populations of *A. halleri* that were reported by Sitko et al. (2017). Moreover, an analysis of the phenomenological energy fluxes showed that the PSII of the A-M plants in the field was less damaged compared to the H-M plants (Figure S5). Despite the visible negative effect of TME on the PSII in both M populations, the PSII efficiency was comparable to that in the A-NM population (Figure S5).

In the controlled hydroponic conditions, the A-M and H-M populations had extremely high tolerance of the photosynthetic apparatus to Cd compared to the NM populations (Figure 6b). The A-M population was more sensitive to Cd and had more damage to the photosynthetic apparatus after Cd stress compared to the H-M population (Figure 6b). On the other hand, the end elements of the electron transport chain (ΔH and ΔG) appeared to be undamaged by Cd in both of the M populations compared to the control. We showed that the φP_0 of both of the NM populations was already significantly decreased after treatment with the lowest Cd concentration (50 μM), whereas both of the M populations were much more tolerant (Table S4). Similarly, Szopiński et al. (2019) showed that the photosynthetic apparatus of A-M population was as tolerant to an extremely high Cd and Zn concentration as the H-M population from the same metalliferous site.

4.3 | Flavonols and anthocyanins are important factors in plant tolerance to toxic TME

Flavonols and anthocyanins belong to flavonoids, a large group of widely occurring phenylpropanoid specialized metabolites that are produced by plants (Corso, Perreau, Mouille, & Lepiniec, 2020; Kasprzak, Erxleben, & Ochocki, 2015; Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006; Nakabayashi et al., 2014; Tohge, de Souza, & Fernie, 2017). The ability of flavonoids to scavenge reactive oxygen species (ROS) and thus to protect plants from the oxidative stress that is caused by different stress factors has been documented in many studies (Corso et al., 2018; Kasprzak et al., 2015; Skórzyńska-Polit, Drążkiewicz, & Krupa, 2010). Moreover, it was reported that in vitro, flavonoids are able to form complexes with TME, which suggests that they have an additional function as metal chelator agents (Kasprzak et al., 2015). However, further investigations are needed to fully characterize the role of flavonols and anthocyanins in plants that have been exposed to TME stress.

The H-M and A-M populations had a constitutive and higher content of flavonol and anthocyanin compared to the NM populations in both the field (Figure 3b,c) and hydroponic conditions (Figure 7b,c), which may be linked to their adaptation to a high concentration of TME *in situ* as well as the higher tolerance of these populations. The significantly higher flavonol content index in the A-M plants and the anthocyanin content index in the H-M plants from Piekary Śląskie (metalliferous) suggest contrasting responses to the stress that is caused by the presence of high TME concentrations in both species.

In the controlled conditions, both the A-M and H-M populations had a higher flavonol content index in the untreated plants compared to the A-NM and H-NM populations, which may be indicative of a constitutively higher biosynthesis of these specialized metabolites in the M populations (Figure 7b). In strong support of this, Corso et al. (2018) reported a constitutively higher expression of several of the genes that are involved in the biosynthesis of flavonoids as well as a higher accumulation of the associated metabolites in an *A. halleri* Cd and Zn hyperaccumulator population from Poland. They also showed that the *A. thaliana* knockout mutants for the flavonoid-biosynthesis genes were more sensitive to Cd compared to the wild-type plants (Corso et al., 2018). Moreover, Skórzyńska-Polit et al. (2004) demonstrated a high increase in the content of flavonols in the plants of *Phaseolus coccineus* that had been treated with Cd compared to the control plants.

The Cd treatments caused an increase in the anthocyanin content index in all of the investigated populations, however, in the NM populations, this increase was significantly higher compared to the M populations (Figure 7c). The lower content of anthocyanins in the M populations compared to the NM populations suggests a higher tolerance of the A-M and H-M populations. Skórzyńska-Polit et al. (2010) reported that Cd (25 and 50 μM) significantly increased the anthocyanin content in the leaves of *A. thaliana* compared to untreated plants. A considerable increase in the anthocyanin content index was also demonstrated by Szopiński et al. (2019) for A-M and H-M populations after 1.0 mM Cd and 5.0 mM Zn treatment for 5 days. In addition, there have also been reports that the application of exogenous anthocyanins reduced the negative effects of TME, which further support the role of these pigments in the plant defence against metal toxicity (Ahmed, Salih, & Hadi, 2013; Glińska et al., 2007).

5 | CONCLUSIONS

This study presents an in-depth physiological, ionomic and pigment accumulation comparison of *A. halleri* and *A. arenosa* species from metalliferous and non-metalliferous sites in Southern Poland. The ionomic data, pigment content indices and an extensive analysis of the photosynthetic apparatus of two *A. arenosa* and two *A. halleri* populations, both *in situ* and in controlled conditions, provide evidence that the two species evolved partially different strategies for the local adaptation to TME-contaminated sites. These differences include regulating the photosynthetic apparatus, the profiles of flavonol and anthocyanin and the accumulation of Cd and Zn. Moreover, the data obtained from our research shows that the M ecotype of *A. arenosa* has a hypertolerance to Cd and Zn. While NM *A. halleri* population constitutively hyperaccumulates Cd and Zn, the hyperaccumulation in *A. arenosa* appeared to be dependent on the

high concentration of TME in the growth substrate, which resembled the behaviour of indicator plants. We believe that more in-depth molecular studies that focus on an interspecific comparison between *A. halleri* and *A. arenosa* and their local adaptation to M sites will greatly benefit our understanding of the mechanisms that are involved in homeostasis of TME in plants.

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AUTHORS' CONTRIBUTIONS

Michał Szopiński, Krzysztof Sitko, Nathalie Verbruggen and Eugeniusz Małkowski conceived and designed the research. Michał Szopiński, Krzysztof Sitko, and Magdalena Rojek-Jelonek conducted the experiments. Michał Szopiński, Krzysztof Sitko, Szymon Rusinowski, Paulina Zieleźnik-Rusinowska, Massimiliano Corso, Magdalena Rojek-Jelonek, and Adam Rostański analyzed the data. Michał Szopiński wrote the first draft of the manuscript, which was extensively edited by all of the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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8. CHAPTER II: Toxic effects of Cd and Zn on the photosynthetic apparatus of the *Arabidopsis halleri* and *Arabidopsis arenosa* pseudo-metallophytes

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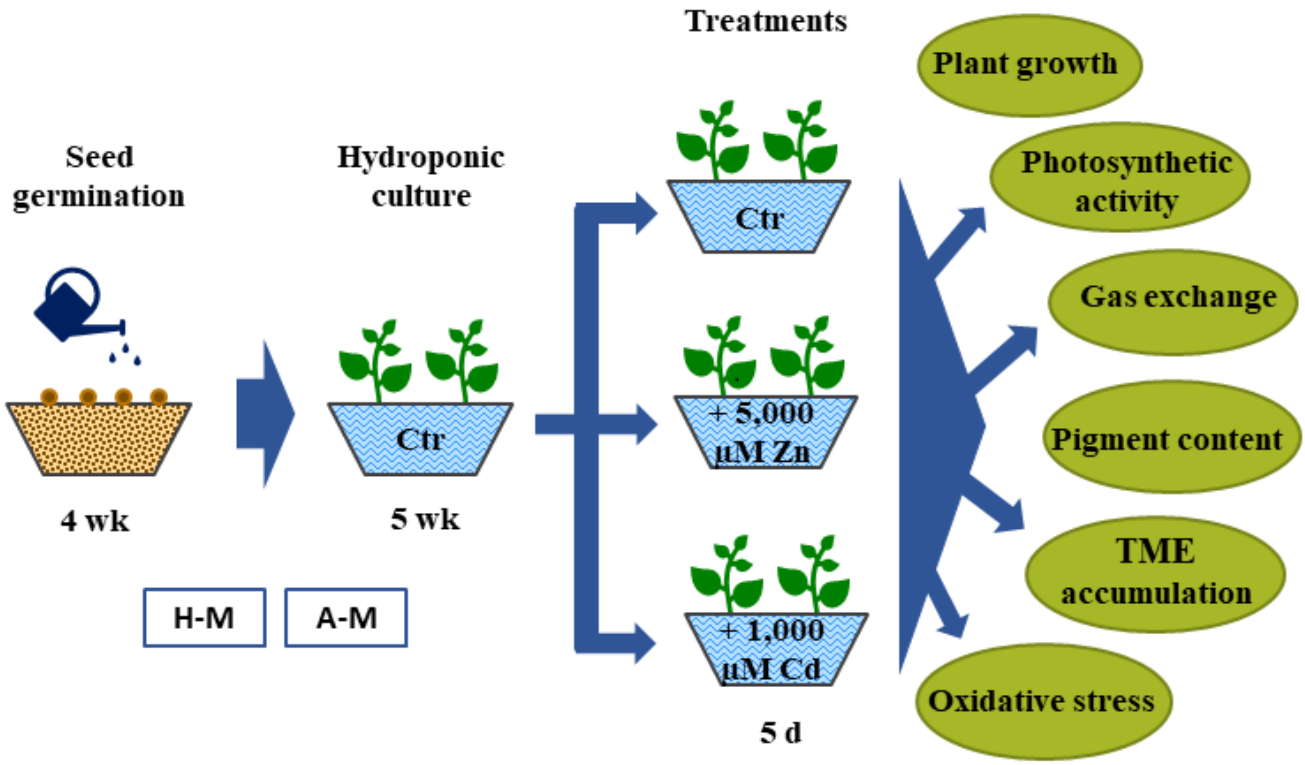

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Toxic Effects of Cd and Zn on the Photosynthetic Apparatus of the *Arabidopsis halleri* and *Arabidopsis arenosa* Pseudo-Metallophytes

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Hyperaccumulation and hypertolerance of Trace Metal Elements (TME) like Cd and Zn are highly variable in pseudo-metallophytes species. In this study we compared the impact of high Cd or Zn concentration on the photosynthetic apparatus of the *Arabidopsis arenosa* and *Arabidopsis halleri* pseudo-metallophytes growing on the same contaminated site in Piekary Slaskie in southern Poland. Plants were grown in hydroponic culture for 6 weeks, and then treated with 1.0 mM Cd or 5.0 mM Zn for 5 days. Chlorophyll *a* fluorescence and pigment content were measured after 0, 1, 2, 3, 4, and 5 days in plants grown in control and exposed to Cd or Zn treatments. Moreover, the effect of TME excess on the level of oxidative stress and gas-exchange parameters were investigated. In both plant species, exposure to high Cd or Zn induced a decrease in chlorophyll and an increase in anthocyanin contents in leaves compared to the control condition. After 5 days Cd treatment, energy absorbance, trapped energy flux and the percentage of active reaction centers decreased in both species. However, the dissipated energy flux in the leaves of *A. arenosa* was smaller than in *A. halleri*. Zn treatment had more toxic effect than Cd on electron transport in *A. halleri* compared with *A. arenosa*. *A. arenosa* plants treated with Zn excess did not react as strongly as in the Cd treatment and a decrease only in electron transport flux and percentage of active reaction centers compared with control was observed. The two species showed contrasting Cd and Zn accumulation. Cd concentration was almost 3-fold higher in *A. arenosa* leaves than in *A. halleri*. On the opposite, *A. halleri* leaves contained 3-fold higher Zn concentration than *A. arenosa*. In short, our results showed that the two *Arabidopsis* metallicolous populations are resistant to high Cd or Zn concentration, however, the photosynthetic apparatus responded differently to the toxic effects.

Keywords: photosystem II, Cadmium, Zinc, Arabidopsis, chlorophyll *a* fluorescence

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INTRODUCTION

Due to industrial and agricultural activities, such as mining, smelting, traffic, using of fertilizers, and sewage sludges, as well as natural processes including atmospheric deposition and weathering of minerals metal contamination has become serious environmental problem worldwide (Alloway, 2013; Su et al., 2014).

Cadmium (Cd) is considered as one of the most toxic non-essential elements for plants (Clemens and Ma, 2016). Despite zinc (Zn) being an essential microelement (Kabata-Pendias, 2011), its excess in plants can also induce phytotoxic effects (Chaney, 1993). It was found that excess of both trace metal elements (TME) can have similar negative influence on different elements of photosynthetic apparatus, for example: pigment biosynthesis, light capture, electron transport, stomatal conductance, CO_2 assimilation, and activity of enzymes in Calvin cycle (Clijsters and Van Assche, 1985; Van Assche and Clijsters, 1986; Myśliwska-Kurdziel et al., 2002; Sagardoy et al., 2010; Vassilev et al., 2011; Verbruggen et al., 2013). However, the toxic effects of high Cd and excess Zn concentration on the complex process of photosynthesis are still poorly understood (Paunov et al., 2018).

Arabidopsis halleri and *A. arenosa* are pseudo-metallophytes closely related to *A. thaliana* (Clauss and Koch, 2006), which are used to study the adaptation to environments highly contaminated with TME. Both species can be commonly found on metalliferous and non-metalliferous sites in southern Poland (Fiałkiewicz and Rostanski, 2006; Szarek-Łukaszewska and Grodzinska, 2007, 2011; Preite et al., 2018). It was documented that metalliferous populations of *A. halleri* and *A. arenosa* are hypertolerant to Cd and Zn (Przedpełska and Wierzbicka, 2007; Nadgórska-Socha et al., 2013; Sitko et al., 2017; Stein et al., 2017). While Zn hyperaccumulation is a constitutive trait, Cd accumulation is highly variable within *A. halleri* populations (Pauwels et al., 2012; Meyer et al., 2015; Sitko et al., 2017; Stein et al., 2017; Corso et al., 2018; Frérot et al., 2018; Schvartzman et al., 2018), whereas these traits are poorly studied in *A. arenosa*.

Measurements of chlorophyll *a* fluorescence is a noninvasive and sensitive method for monitoring physiological status of plants (Baker, 2008; Kalaji et al., 2012, 2014; Sitko et al., 2017). In recent years such measurements have become more and more commonly used under various conditions such as TME stress (Kalaji et al., 2014; Daszkowska-Golec et al., 2017; Sitko et al., 2017; Paunov et al., 2018). Analysis of chlorophyll *a* fluorescence kinetics, known as OJIP transient, can provide information on electron transport reactions mainly inside PSII and parts of PSI (Baker, 2008; Kalaji et al., 2014; Goltsev et al., 2016; Paunov et al., 2018). In order to obtain more complete picture of effect of TME excess on photosynthesis, measurements of leaf gas-exchange parameters such as photosynthetic rate based on CO_2 assimilation and parameters based on chlorophyll *a* fluorescence are necessary (Arshad et al., 2015; Rusinowski et al., 2019).

Measurements of pigment contents such as chlorophyll, flavonols and anthocyanins have also become increasingly popular due to the development of portable devices, which enable measurements of these pigment contents in laboratory as well as *in situ* (Cerovic et al., 2015; Lefebvre et al., 2016; Gonzalez-Mendoza et al., 2017; Hosseini et al., 2017; Sitko et al., 2017). Flavonols and anthocyanins belong to flavonoid secondary metabolites, which are the largest class of polyphenols (around 8,000 metabolites) in plants (Mattivi et al., 2006; Tohge et al., 2017). Flavonoids are characterized by two benzene rings linked by a heterocyclic pyran ring and primarily occurring in plants as O-glycosides. They play a major role in plant protection against negative effects of abiotic and biotic stress factors in model and crop plants (Jaakola et al., 2004; Corso et al., 2015; Tohge et al., 2017). Flavonols and anthocyanins can serve as protection against damage caused by TME, free oxygen radicals and excessive light radiation (Gould, 2004; Emiliania et al., 2013; Landi et al., 2015; Peng et al., 2017; Tohge et al., 2017; Moustaka et al., 2018).

High concentrations of TME such as Cd and Zn can interfere with numerous physiological processes in plants. As a result the overproduction of reactive oxygen species (ROS) such as H_2O_2 occurs and oxidative stress in plants is observed (Sandalio et al., 2009; Moura et al., 2012). One of the enzymes, which is responsible for the decomposition of H_2O_2 is catalase. For this reason, the changes in activity of this enzyme are used as a marker of plant resistance to oxidative stress (Moura et al., 2012; Rusinowski et al., 2019). When excess of ROS and/or H_2O_2 appeared in plants and oxidative stress develops, lipid peroxidation takes place. One of the products of lipid degradation is malondialdehyde (MDA). Thus, the increase in the content of this chemical compound in plant tissues is frequently taken into account as a measure of oxidative stress level (Bouazizi et al., 2010; Rusinowski et al., 2019).

The aim of this study was to compare the response of the photosynthetic apparatus of two hypertolerant plant species to high concentration of Cd or Zn living on the same contaminated site. In this work we compared physiological responses of two metallicolous *A. arenosa* and *A. halleri* populations from the contaminated site of Piekary Slaskie in South Poland. Presented in the current paper results for chlorophyll *a* fluorescence indicate that *A. arenosa* shows similar tolerance to Cd and Zn as *A. halleri*.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Plants of *Arabidopsis halleri* and *Arabidopsis arenosa* were grown hydroponically in controlled greenhouse conditions. Seeds of both populations were collected from the same metalliferous site in Piekary Slaskie (50°22′00.6"N, 18°58′18.4"E). Vernalized seeds were sown onto vermiculite watered with deionized water for the first 2 weeks and for the next 2 weeks with 1/10 strength Hoagland solution. Four-week-old seedlings were transferred into hydroponic containers (three seedlings of each species per container) filled with 2 L of ½ strength Hoagland solution (Bloom, 2006) with initial pH adjusted to 5.8 ± 0.05. Seedlings were grown in a greenhouse under artificial light with high pressure sodium lamps (HPS), photoperiod 12 h light (150 μE m^{-2} s^{-1})/12 h darkness. The temperature in the greenhouse was 20 ± 1°C and air humidity was 60 ± 5%. The medium was

changed twice a week. Plants were grown for 6 weeks in the control solution. After 6 weeks of growth containers were divided into 3 experimental groups: Control (2 containers, 6 plants per species), Cd treatment (3 containers, 9 plants per species), and Zn treatment (3 containers, 9 plants per species). Cd was added at the concentration of 1,000 μM and Zn at the concentration of 5,000 μM, two concentrations that are lethal for non-tolerant plant species, as *Arabidopsis thaliana*. These concentrations were chosen in order to induce toxic effect on photosynthetic apparatus. The concentrations were chosen on the basis of preliminary studies, where half of mentioned concentrations did not show toxic effect on photosynthetic apparatus performance in short-term experiment (data not shown). The experiment was finished after 5 days and all measurements were made after 0, 1, 2, 3, 4, and 5 days, except growth parameters (shoot and root fresh weight, and root length) and accumulation of TME, which was performed on plant material harvested at the end of experiment. The experiment was repeated three times.

Measurements of Plant Growth

At the end of the experiment, plants were harvested and root length was measured. Afterwards, shoot and root fresh biomasses were measured separately.

Analysis of Cd and Zn Accumulation in Leaves

At the end of the experiment leaves of plants were harvested and dried at 80°C for 72 h. Dry plant material was ground in the mortar and subsequently digested in a microwave-assisted wet digestion system (ETHOS 1, Milestone, Italy) according to the procedure provided by the manufacturer (concentrated HNO_3 and 30% H_2O_2, 4:1 v/v). The Cd and Zn concentrations in leaves were analyzed in the digests using flame atomic absorption spectrophotometer (iCE 3,500 FAAS, Thermo Scientific, USA). Reference plant material (Oriental Basma Tobacco Leaves (INCT-OBTL-5), Institute of Nuclear Chemistry and Technology, Poland) was used for the quality assurance of the analytical data.

Measurements of Chlorophyll *a* Fluorescence and Pigment Index

Measurements were done on fully developed *Arabidopsis halleri* and *A. arenosa* leaves which entirely filled the area of the sensor. Chlorophyll *a* fluorescence was measured at 0, 1, 2, 3, 4, and 5 days using the Plant Efficiency Analyzer (PocketPEA fluorimeter, Hansatech Instruments Ltd., England). Before measurement, each selected leaf was adapted in the dark for 30 min using dedicated leaf clips. After adaptation, a saturating light pulse of 3,500 $\mu E\ m^{-2}\ s^{-1}$ was applied for 1 s, which closed all the reaction centers, and the fluorescence parameters were measured. Measurements were done without damaging the plant material.

Chlorophyll, flavonol, and anthocyanin index were measured with the use of Dualex Scientific+ sensor (Force-A, France). Measurements of pigment index were performed at 0, 1, 2, 3, 4, and 5 days on the same leaves as for chlorophyll *a* fluorescence measurements. Measurements were done without damaging the plant material.

Gas-Exchange Parameters

Plant gas exchange parameters, such as intracellular CO_2 concentration (*Ci*), photosynthetic rate (*A*), stomatal conductance (g_s), and transpiration rate (*E*) were conducted on fully developed leaves. Measurements were carried out at the end of experiment, after 5 days using an infrared gas analyzer with special chamber for *Arabidopsis* (LCpro+, ADC Bioscientific, UK) under controlled climate conditions (T = 24°C, Ambient light PAR = 150 $\mu mol\ m^{-2}\ s^{-1}$). Measurements were performed at noon.

Oxidative Stress Parameters

Hydrogen peroxide concentration was determined as described by Bouazizi et al. (2010) with minor modifications. Fresh leaf tissues (150 mg) were homogenized in 1.5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 *g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml potassium phosphate buffer (10 mM, pH 7.0) and 1 ml potassium iodide (KI) (1 M). The absorbance of the supernatant was measured at 390 nm, and the content of H_2O_2 was obtained using a standard curve.

The level of lipid peroxidation estimated by malondialdehyde (MDA) concentration was determined as described by Bouazizi et al. (2010) with minor modifications. Fresh leaf tissues (150 mg) were homogenized in 4 ml 0.25% thiobarbituric acid (TBA) prepared in 10% TCA. The homogenate was incubated in a water bath at 95°C for 30 min and then cooled in an ice bath. After centrifugation at 10,000 *g* for 10 min, the absorbance of the supernatant was measured at 532 nm and corrected by subtracting the non-specific absorbance at 600 nm. Concentration of malondialdehyde (MDA) was calculated using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

The activity of catalase was determined as described by Bouazizi et al. (2010) with minor modifications. Fresh leaf tissues (150 mg) were homogenized in 1.5 ml potassium phosphate buffer (10 mM, pH 7.0). The homogenate was centrifuged at 12,000 *g* for 20 min. The supernatant (0.02 ml) was added to 2 ml 10 mM peroxide prepared in potassium phosphate buffer. Catalase activity was determined spectrophotometrically by monitoring the changes of absorbance caused by H_2O_2 reduction at 240 nm.

Statistical Analysis

The statistically significant differences among mean values were determined using one-way ANOVA and *post hoc* Tukey HSD test ($P < 0.05$). Additionally two-way ANOVA analysis was performed to determine effect of treatment (Ctr, 1.0 mM Cd and 5.0 mM Zn), species (*A. arenosa* and *A. halleri*) as well as interaction between these two factors on measured physiological parameters (**Table S1**). The statistical analysis was performed using the computer software Statistica v.13.1 (Dell Inc., USA). The pipeline models of energy fluxes through leaf's cross section were done using CorelDRAW X6 (Corel Corp., Canada).

RESULTS

Cd and Zn Content in *A. halleri* and *A. arenosa* Leaves

Cadmium and zinc concentrations were measured in leaves of both plant species (**Figure 1**). Remarkably, *A. arenosa* accumulated 3-fold more Cd in the leaves with respect to *A. halleri* (6 and 2 g Cd kg^{-1} DW in *A. arenosa* and *A. halleri*, respectively) (**Figure 1A**). In contrast, *A. halleri* leaves accumulated 13 g Zn kg^{-1} DW, which was 3-fold more compared to Zn accumulated by *A. arenosa* (**Figure 1B**).

Plant Growth

In our control conditions *A. arenosa* plants grew better than *A. halleri*, with a biomass over 2-fold higher than *A. halleri* (**Figures 2A,B**). The biomass of *A. arenosa* shoots was more affected by Cd treatment (60% of control) than by Zn (78% of the control), whereas the biomass of *A. halleri* shoots was similarly affected, irrespective of the metal (53% of the control) (**Figure 2A**). The root biomass in Cd treatment was significantly lower only in *A. arenosa* (23% of the control), whereas Zn treatment considerably diminished root growth (33% of the control) of both species (**Figure 2B**). Only plants of *A. arenosa* had their root length significantly lowered (73% of the control) under Zn treatment (**Figure S1**). Pictures of plants at the end of experiment are presented in **Figure S2**. Two-way ANOVA analysis showed that both treatment type and species significantly influenced shoot and root biomass, however, interaction between treatment type and species was significant only for root biomass (**Table S1**).

Oxidative Stress

Concentrations of H_2O_2 and MDA were significantly increased by Cd treatment in leaves of both species, but not Zn (**Figures 3A,B**). The treatment type had significant impact on both H_2O_2 and MDA concentration, whereas species had significant impact only on MDA concentration. There was no significant interaction between species and treatment for both parameters (**Table S1**).

Catalase activity was lowered by Cd and Zn treatments in leaves of *A. arenosa* compared with control, but not in in *A. halleri* (**Figure 3C**). Both factors (species and treatment) had significant influence on catalase activity, moreover effect of treatment was dependent on the species (**Table S1**).

Pigment Indices

The chlorophyll index in *A. arenosa* and *A. halleri* was similar after 5 days Cd or Zn treatments compared to their respective controls (**Figure 4A**).

The flavonol and anthocyanin index was significantly higher in leaves of *A. halleri* compared to *A. arenosa* in control conditions (**Figures 4B,C**). After 5 days Cd treatment the flavonol index substantially decreased in leaves of *A. arenosa* compared to the control, which was not observed in *A. halleri*. In marked contrast, Zn treatment did not affect the flavonol index of *A. arenosa* leaves, whereas it decreased by 50% the one of *A. halleri* in comparison to the control (**Figure 4B**). Five days Cd treatment caused similar significant increase in the anthocyanin index of *A. arenosa* and *A. halleri* leaves compared with the control, whereas anthocyanin index in Zn treated plants was increased only in *A. arenosa* (**Figure 4C**).

Both factors (species and treatment) had significant influence on chlorophyll and flavonol indices, whereas anthocyanin index was differentiated only by treatment (**Table S1**).

Changes over time in the chlorophyll, flavonol and anthocyanin contents during 5 days treatments are presented in supplementary material (**Figure S3**).

Photosynthetic Apparatus Performance

The parameters describing photosynthesis performance are listed in **Table S2**. Photosynthetic apparatus performance was differently affected by Cd in *A. arenosa* and *A. halleri* (**Figures 5A,B**). Minimal fluorescence (F_0) gradually increased in *A. halleri* treated with Cd, whereas it was not affected in *A. arenosa* in the same conditions (**Table 1**). The presence of the positive ΔK-band in ΔV_t curves in *A. arenosa* may be correlated with damage or uncoupling of Oxygen Evolving Complex (OEC) caused by Cd treatment (**Figure 5A**). Toxic effect of Cd in point ΔJ was observed in *A. arenosa* after 48 h of treatment and it remained unchanged till the end of experiment, whereas no significant changes were observed for ΔI compared with the control. Changes observed between ΔJ-ΔI can be attributed to the reduced rate of electron transfer between quinone acceptors (from Q_A to Q_B). Slight increase in values compared with the control in points ΔH and ΔG observed in *A. arenosa* treated with Cd can be connected with damage to plastoquinone pool and PSI end of electron acceptors such as Ferredoxin-$NADP^+$ Reductase (FNR). In contrast to *A. arenosa*, Cd seemed to stimulate performance of photosynthetic apparatus of *A. halleri* compared with control, with slight negative effect observed only after 5 days in points ΔK and ΔH (**Figure 5B**). Maximal fluorescence (F_m) in *A. arenosa* gradually decreased during Cd exposure and was significantly lower after 4 days, whereas F_m in *A. halleri* significantly decreased only after 5 days (**Table 1**). Zn treatment affected differently *A. arenosa* and *A. halleri* performance of photosynthetic apparatus. Minimal fluorescence (F_0) was not affected by Zn treatment in *A. arenosa* (**Table 1**), whereas in *A. halleri* F_0 increased considerably with the exposure to high concentration of Zn (**Table 1**). *A. arenosa* was more affected by Zn treatment compared with *A. halleri* (**Figures 5C,D**). Toxic effect of Zn excess in *A. arenosa* compared with control was observed earlier than in *A. halleri*. Moreover, in *A. halleri* at the beginning of Zn treatment slight stimulation of photosynthetic apparatus was observed and at the end of the experiment the toxic effect was not as high as in *A. arenosa* (**Figures 5C,D**). F_m was significantly lowered in *A. arenosa* after 5 days of Zn treatment, whereas in *A. halleri* this parameter was not affected by the treatment (**Table 1**).

Parameters describing characteristics of photosynthetic apparatus in general did not significantly change for both species in the control (**Table 1**). Changes observed in chlorophyll *a* fluorescence kinetics for Cd treated plants were confirmed by the value of maximum quantum efficiency of the PSII (ϕP_0) which decreased with the trace metal elements (TME) exposure

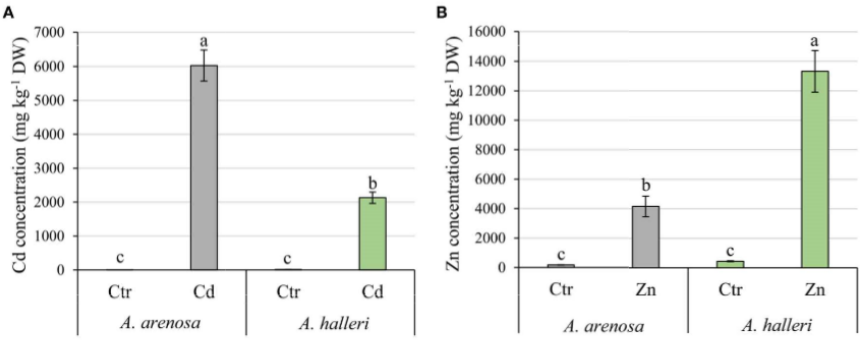

FIGURE 1 | Accumulation of Cd **(A)** and Zn **(B)** in *A. arenosa* (gray) and *A. halleri* (green) leaves after 120 h of experiment. Ctr—control; Cd—1.0 mM Cd treatment; Zn—5.0 mM Zn treatment. Values are means ± SE ($n = 6$). Means followed by the same letter for each metal treatment are not significantly different from each other using the HSD test ($P < 0.05$).

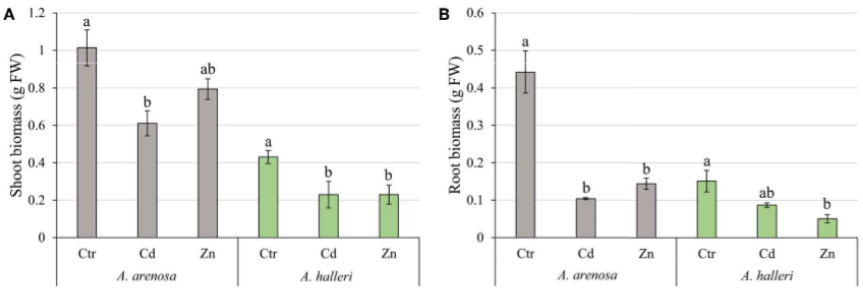

FIGURE 2 | Growth of the shoots **(A)** and roots **(B)** of *A. arenosa* (gray) and *A. halleri* (green) after 120 h of experiment. Ctr—control; Cd—1.0 mM of Cd treatment; Zn—5.0 mM of Zn treatment. Values are means ± SE ($n = 6$). Means followed by the same letter for each species are not significantly different from each other using the HSD test ($P < 0.05$).

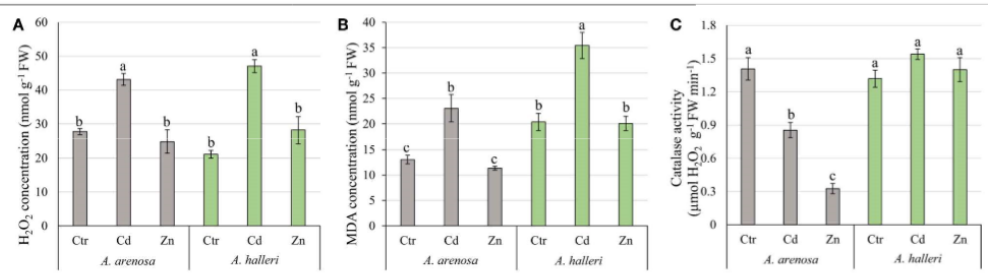

FIGURE 3 | Concentration of H_2O_2 **(A)**, MDA **(B)**, and catalase activity **(C)** in shoots of *A. arenosa* (gray) and *A. halleri* (green) after 120 h of experiment. Ctr—control; Cd—1.0 mM Cd treatment; Zn—5.0 mM Zn treatment. Values are means ± SE ($n = 6$). Means followed by the same letter are not significantly different from each other using the HSD test ($P < 0.05$).

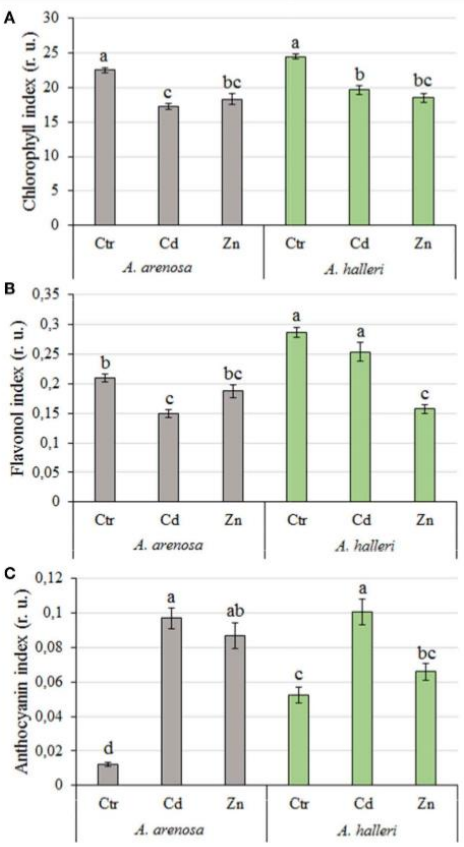

FIGURE 4 | Chlorophyll **(A)**, flavonol **(B)**, and anthocyanin **(C)** index (relative units) in *A. arenosa* (gray) and *A. halleri* (green) leaves after 120 h of experiment. Ctr—control; Cd—1.0 mM Cd treatment; Zn—5.0 mM Zn treatment. Values are means ($n = 30$). Means followed by the same letter are not significantly different from each other, using Tukey HSD test ($P < 0.05$).

time (**Table 1**). Parameters such as quantum yield (ϕE_0) and probability for electron transport from reduced plastoquinone (Q_A^-) to plastoquinone (ψE_0) significantly decreased toward the end of the experiment only in *A. halleri*. Moreover, the quantum yield (ϕR_0) and probability for the reduction of the end electron acceptors at the PSI acceptor side (δR_0) were decreased in both species over the duration of Cd treatment. Quantum yield of energy dissipation (ϕD_0) increased in both species under Cd treatment (**Table 1**). ϕP_0 of *A. arenosa* was not affected by the Zn treatment, whereas in *A. halleri* it was significantly lowered at the end of the experiment (**Table 1**). ϕE_0 and ψE_0 in *A. arenosa* were considerably decreased after 1 days, whereas in *A. halleri* the significant decrease in these parameters was observed at the end of experiment (5 days). δR_0 was not substantially changed over time in both species treated with Zn, whereas ϕR_0 was significantly lowered after 1 and 3 days of Zn treatment in *A. arenosa* and *A. halleri*, respectively. ϕD_0 did not change during the Zn treatment, however, in *A. halleri* it significantly increased only after 5 days (**Table 1**).

Overall efficiency of PSII, showed as models of phenomenological energy fluxes per cross section (CS) of *A. arenosa* and *A. halleri*, was differently affected by Cd or Zn treatment after 5 days compared with the control (**Figure 6**). After 5 days of Cd treatment absorption flux (ABS/CS) was considerably lower in both species compared with the control, whereas Zn treatment did not cause a significant change in both species (**Figure 6**). Trapped energy flux was significantly diminished by Cd and Zn treatment in both species, except in *A. arenosa* treated with Zn. Electron transport flux (ET/CS) was not lowered compared with the control only in *A. halleri* treated with Cd. Both Cd and Zn treatment caused significant increase in percentage of inactive reaction centers (RC) in *A. arenosa*, whereas in *A. halleri* significant increase in this parameter was observed only in Cd treatment (**Figure 6**).

Gas-Exchange Parameters

The concentration of intercellular CO_2 (C_i) was considerably decreased by 5 days Cd treatment in *A. arenosa* and *A. halleri* (**Figure 7A**). In control conditions the photosynthetic rate (A) of *A. arenosa* was more than twice that of *A. halleri* (**Figure 7B**). Both Cd ad Zn treatments caused a considerable decrease of the photosynthetic rate only in *A. arenosa* (38 and 49% of control for Cd and Zn, respectively) after 5 days (**Figure 7B**). In control conditions the transpiration rate (E) of *A. arenosa* was twice that of *A. halleri* (**Figure 7C**). Cd treatment caused a significant decrease in E for both species (19 and 30% of the control for *A. arenosa* and *A. halleri*, respectively). On the contrary, E significantly decreased during Zn treatment only in *A. arenosa* (35% of the control) (**Figure 7C**). In the control conditions, the stomatal conductance (g_s) of *A. arenosa* was more than twice that of *A. halleri* (**Figure 7D**). A significant decrease in g_s was observed in Cd treated *A. arenosa* (12% of the control) and *A. halleri* (26% of the control). In Zn treated plants, significant decrease in g_s was observed only in *A. arenosa* (22% of the control) (**Figure 7D**). All gas-exchange parameters were considerably affected by both treatments and species. Moreover, interaction between treatment type and species was not substantial only for C_i (**Table S1**).

DISCUSSION

In order to gain a better understanding of mechanisms underlying metal tolerance of *A. halleri* and *A. arenosa*, metal content, photosynthetic activity, levels of oxidative stress, gas-exchange parameters, chlorophyll, flavonol, and anthocyanin indices were analyzed in metallicolous populations from the same contaminated site in southern Poland grown in control, and exposed to high Cd and Zn concentrations.

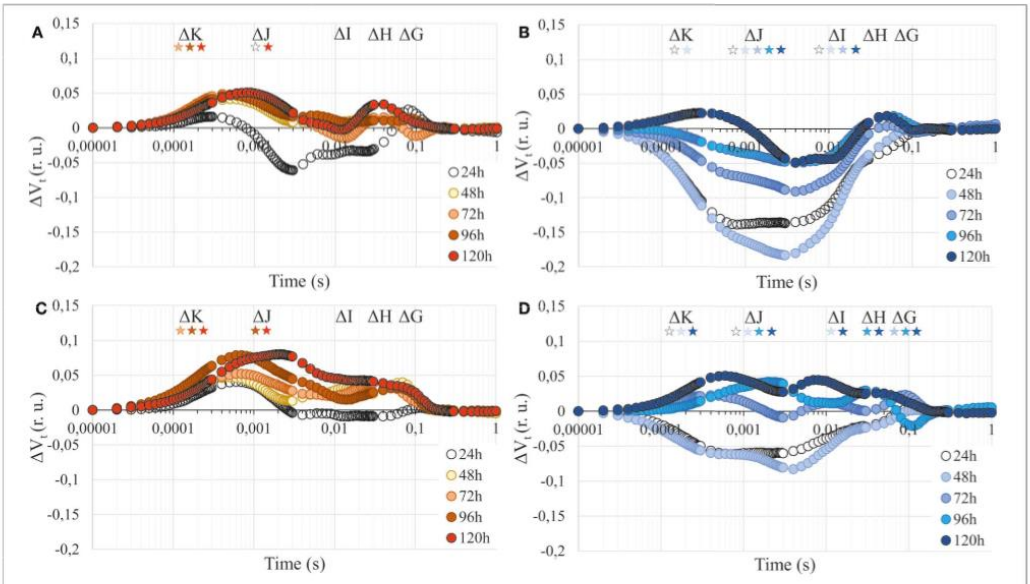

FIGURE 5 | Chlorophyll *a* fluorescence induction curves presented as relative variable fluorescence (ΔV_t) after 24, 48, 72, 96, and 120 h of experiment. **(A)**—*A. arenosa* treated with 1.0 mM Cd, **(B)**—*A. halleri* treated with 1.0 mM Cd, **(C)**—*A. arenosa* treated with 5.0 mM Zn, **(D)**—*A. halleri* treated with 5.0 mM Zn. For ΔV_t analysis, the fluorescence of control was the reference and equaled 0. Values are means ($n = 8$). Asterisks indicate statistically significant differences (Fisher's LSD test, $P < 0.05$) between fluorescence of plants treated with HM and the control for each band (ΔK, ΔJ, ΔI, ΔH, and ΔG). Asterisk are arranged chronologically from 24 to 120 h and asterisks color corresponds to point color, which represent the time of metal treatment. (r. u.), relative units.

Cd and Zn contents in leaves (**Figures 1A,B**) suggest that the two species differ in the mechanisms involved in the uptake and transport of these metals, with a clear preference for Zn accumulation in *A. halleri* and for Cd accumulation in *A. arenosa*. Since there are no reports, which compare both plant species in control conditions it is impossible to explain these differences. However, it is tempting to suggest that the higher accumulation of Zn by *A. halleri* and higher accumulation of Cd by *A. arenosa* is connected with different transport activity of transporters responsible for the uptake and translocation of both metals in plants. In order to elucidate this deference between the species further research on gene expression levels are needed. Nevertheless, our results showed that the studied metallicolous populations of *A. arenosa* and *A. halleri* are capable of accumulating high concentrations of Cd and Zn. Moreover, the concentrations of Cd and Zn in *A. arenosa* observed in the current study are comparable to the concentrations reported in hyperaccumulator plant species by other authors (Meyer et al., 2010; Farinati et al., 2011; Corso et al., 2018; Schvartzman et al., 2018).

We observed that high Cd treatment (1.0 mM) decreased the growth of shoots in both *A. arenosa* and *A. halleri* (**Figure 2A**). Despite the higher accumulation of Cd in *A. arenosa* (**Figure 1A**), the decrease in shoot biomass was slightly different (60% of control) than that observed in *A. halleri* (53% of control). This finding shows high tolerance to Cd of *A. arenosa* and *A. halleri*. In plants treated with high Zn concentration (5.0 mM), the shoot biomass was considerably lowered only in *A. halleri*, but it is important to note that accumulation of Zn in *A. halleri* was almost three-times higher than in *A. arenosa* (**Figure 1B**).

Our results showed that Cd caused similar and significant increase in the concentrations of hydrogen peroxide and MDA, while Zn treatment did not cause any change compared to the control conditions in *A. arenosa* and *A. halleri* (**Figures 3A,B**). However, considering contrasting accumulation of Cd and Zn in both species, our data suggests that anti-oxidative mechanisms in *A. arenosa* can better cope with Cd-induced oxidative stress compared to *A. halleri*, while the opposite trend was observed for Zn. Furthermore, large difference in catalase activity in the response to Cd or Zn treatment was found between *A. arenosa* and *A. halleri*, which suggests that various anti-oxidative mechanisms may function in both species. Cd induced oxidative stress in *A. halleri* has been already reported by other authors (Baliardini et al., 2015; Ahmadi et al., 2018).

Measurements of chlorophyll content can be useful for assessing plant tolerance to stress (Rong-hua et al., 2006; Ramirez et al., 2014; Xue et al., 2018). Relatively low alterations in chlorophyll content measured after exposure to

TABLE 1 | Characteristics of the photosynthetic apparatus.

Treatment	Species	Time (h)	F_0	F_m	ϕD_0	ϕP_0	ΨE_0	ϕE_0	δR_0	ϕR_0
Control	*A. arenosa*	0	5650 ± 160*ab*	30500 ± 370*b*	0.18 ± 0.00*ab*	0.82 ± 0.00*ab*	0.47 ± 0.01*ab*	0.38 ± 0.01*ab*	0.32 ± 0.02*a*	0.12 ± 0.01*a*
		24	6470 ± 430*a*	32900 ± 650*ab*	0.20 ± 0.01*a*	0.80 ± 0.01*b*	0.44 ± 0.02*b*	0.35 ± 0.02*b*	0.28 ± 0.02*a*	0.10 ± 0.01*a*
		48	5650 ± 60*ab*	32300 ± 350*ab*	0.17 ± 0.00*ab*	0.83 ± 0.00*ab*	0.50 ± 0.01*ab*	0.41 ± 0.01*ab*	0.32 ± 0.01*a*	0.13 ± 0.01*a*
		72	5640 ± 140*ab*	32100 ± 400*ab*	0.18 ± 0.00*ab*	0.82 ± 0.00*ab*	0.48 ± 0.01*ab*	0.40 ± 0.01*ab*	0.32 ± 0.01*a*	0.13 ± 0.01*a*
		96	5660 ± 110*ab*	33100 ± 650*a*	0.17 ± 0.00*b*	0.83 ± 0.00*a*	0.51 ± 0.01*a*	0.43 ± 0.01*a*	0.29 ± 0.01*a*	0.12 ± 0.01*a*
		120	5270 ± 120*b*	31000 ± 760*ab*	0.17 ± 0.00*b*	0.83 ± 0.00*a*	0.50 ± 0.02*ab*	0.41 ± 0.01*ab*	0.32 ± 0.01*a*	0.13 ± 0.01*a*
	A. halleri	0	6580 ± 390*b*	30800 ± 490*a*	0.21 ± 0.01*b*	0.79 ± 0.01*a*	0.34 ± 0.02*b*	0.27 ± 0.02*ab*	0.30 ± 0.01*ab*	0.08 ± 0.01*ab*
		24	7130 ± 500*a*	31000 ± 720*a*	0.26 ± 0.02*a*	0.74 ± 0.02*b*	0.28 ± 0.03*b*	0.21 ± 0.03*b*	0.25 ± 0.02*c*	0.06 ± 0.01*b*
		48	6900 ± 270*ab*	32100 ± 610*a*	0.22 ± 0.01*b*	0.78 ± 0.01*a*	0.35 ± 0.02*b*	0.27 ± 0.02*ab*	0.29 ± 0.01*b*	0.08 ± 0.01*ab*
		72	6440 ± 190*b*	32400 ± 560*a*	0.20 ± 0.01*b*	0.80 ± 0.01*a*	0.37 ± 0.01*b*	0.30 ± 0.01*ab*	0.30 ± 0.01*ab*	0.09 ± 0.00*a*
		96	6120 ± 190*b*	32700 ± 620*a*	0.19 ± 0.00*b*	0.81 ± 0.00*a*	0.43 ± 0.01*a*	0.35 ± 0.01*a*	0.31 ± 0.01*a*	0.11 ± 0.01*a*
		120	5970 ± 140*b*	31700 ± 720*a*	0.19 ± 0.00*b*	0.81 ± 0.00*a*	0.40 ± 0.01*ab*	0.33 ± 0.01*a*	0.32 ± 0.01*a*	0.10 ± 0.01*a*
Cd	*A. arenosa*	0	5690 ± 230*a*	31400 ± 1000*a*	0.18 ± 0.00*b*	0.82 ± 0.00*a*	0.55 ± 0.03*a*	0.45 ± 0.02*a*	0.29 ± 0.01*a*	0.13 ± 0.00*a*
		24	5580 ± 240*a*	31800 ± 600*a*	0.18 ± 0.01*b*	0.82 ± 0.01*a*	0.48 ± 0.02*a*	0.40 ± 0.02*a*	0.28 ± 0.01*ab*	0.11 ± 0.00*ab*
		48	6150 ± 360*a*	30300 ± 500*ab*	0.20 ± 0.01*b*	0.80 ± 0.01*ab*	0.48 ± 0.02*a*	0.38 ± 0.02*a*	0.27 ± 0.01*ab*	0.11 ± 0.01*ab*
		72	5600 ± 300*a*	29600 ± 550*ab*	0.19 ± 0.01*b*	0.81 ± 0.01*ab*	0.48 ± 0.01*a*	0.39 ± 0.02*a*	0.25 ± 0.01*b*	0.10 ± 0.01*bc*
		96	5860 ± 400*a*	29000 ± 630*b*	0.20 ± 0.01*ab*	0.80 ± 0.01*b*	0.47 ± 0.02*a*	0.38 ± 0.02*a*	0.25 ± 0.01*b*	0.09 ± 0.01*bc*
		120	6590 ± 540*a*	27400 ± 1360*b*	0.25 ± 0.03*a*	0.75 ± 0.03*b*	0.47 ± 0.03*a*	0.36 ± 0.02*a*	0.22 ± 0.02*b*	0.08 ± 0.01*c*
	A. halleri	0	6130 ± 150*b*	33000 ± 480*a*	0.19 ± 0.00*c*	0.81 ± 0.00*a*	0.55 ± 0.02*ab*	0.45 ± 0.02*ab*	0.30 ± 0.02*ab*	0.13 ± 0.01*a*
		24	5960 ± 70*c*	34800 ± 980*a*	0.17 ± 0.00*c*	0.83 ± 0.00*a*	0.46 ± 0.02*abc*	0.38 ± 0.01*bc*	0.30 ± 0.01*ab*	0.11 ± 0.01*a*
		48	7030 ± 430*b*	32900 ± 320*a*	0.21 ± 0.01*bc*	0.79 ± 0.02*ab*	0.44 ± 0.02*abc*	0.35 ± 0.02*bc*	0.26 ± 0.01*bc*	0.09 ± 0.01*ab*
		72	8110 ± 530*a*	33200 ± 590*a*	0.24 ± 0.02*b*	0.76 ± 0.02*bc*	0.36 ± 0.04*c*	0.28 ± 0.03*c*	0.20 ± 0.02*c*	0.06 ± 0.01*b*
		96	7980 ± 560*ab*	32300 ± 650*ab*	0.25 ± 0.02*ab*	0.75 ± 0.02*bc*	0.38 ± 0.04*c*	0.29 ± 0.04*c*	0.21 ± 0.02*c*	0.07 ± 0.01*b*
		120	7850 ± 550*ab*	29700 ± 590*b*	0.27 ± 0.02*ab*	0.73 ± 0.02*c*	0.45 ± 0.03*bc*	0.34 ± 0.03*bc*	0.22 ± 0.02*bc*	0.08 ± 0.01*b*
Zn	*A. arenosa*	0	6420 ± 250*a*	34400 ± 870*a*	0.19 ± 0.01*a*	0.81 ± 0.01*a*	0.58 ± 0.02*a*	0.47 ± 0.01*a*	0.32 ± 0.02*a*	0.15 ± 0.01*a*
		24	5610 ± 190*a*	31400 ± 500*ab*	0.18 ± 0.01*a*	0.82 ± 0.01*a*	0.49 ± 0.01*b*	0.40 ± 0.01*b*	0.28 ± 0.01*a*	0.11 ± 0.01*b*
		48	6140 ± 180*a*	30400 ± 340*b*	0.20 ± 0.01*a*	0.80 ± 0.01*a*	0.49 ± 0.02*b*	0.39 ± 0.01*b*	0.28 ± 0.02*a*	0.11 ± 0.01*b*
		72	6180 ± 300*a*	32400 ± 780*ab*	0.19 ± 0.01*a*	0.81 ± 0.01*a*	0.46 ± 0.02*b*	0.38 ± 0.02*b*	0.26 ± 0.01*a*	0.10 ± 0.01*b*
		96	6150 ± 340*a*	32000 ± 750*ab*	0.19 ± 0.01*a*	0.81 ± 0.01*a*	0.46 ± 0.02*b*	0.37 ± 0.02*b*	0.26 ± 0.01*a*	0.10 ± 0.01*b*
		120	5890 ± 190*a*	30500 ± 690*b*	0.19 ± 0.01*a*	0.81 ± 0.01*a*	0.46 ± 0.02*b*	0.37 ± 0.02*b*	0.27 ± 0.01*a*	0.10 ± 0.01*b*
	A. halleri	0	6800 ± 340*b*	34200 ± 640*a*	0.20 ± 0.01*b*	0.80 ± 0.01*a*	0.51 ± 0.03*a*	0.40 ± 0.02*a*	0.26 ± 0.01*ab*	0.11 ± 0.00*a*
		24	6330 ± 200*b*	33600 ± 680*a*	0.19 ± 0.00*b*	0.81 ± 0.00*a*	0.40 ± 0.02*b*	0.33 ± 0.02*ab*	0.31 ± 0.01*a*	0.10 ± 0.01*a*
		48	7770 ± 750*ab*	32500 ± 650*a*	0.22 ± 0.02*ab*	0.78 ± 0.02*ab*	0.35 ± 0.03*bc*	0.28 ± 0.03*bc*	0.28 ± 0.01*a*	0.08 ± 0.01*ab*
		72	7830 ± 310*ab*	33700 ± 1220*a*	0.24 ± 0.02*ab*	0.76 ± 0.02*ab*	0.44 ± 0.04*ab*	0.33 ± 0.03*ab*	0.20 ± 0.01*b*	0.07 ± 0.01*bc*
		96	7770 ± 310*ab*	34100 ± 1280*a*	0.23 ± 0.02*ab*	0.77 ± 0.02*ab*	0.46 ± 0.03*ab*	0.35 ± 0.03*a*	0.21 ± 0.01*b*	0.07 ± 0.01*bc*
		120	9550 ± 850*a*	33100 ± 1600*a*	0.30 ± 0.02*a*	0.70 ± 0.03*b*	0.27 ± 0.03*c*	0.19 ± 0.03*c*	0.21 ± 0.01*b*	0.04 ± 0.01*c*

Changes in parameters derived from the chlorophyll a fluorescence signal, describing photosynthetic apparatus of A. arenosa and A. halleri in control and under 1.0 mM Cd or 5.0 mM Zn treatement after 0, 24, 48, 72, 96, and 120 h. Values are means ± SE (n = 8). Means followed by the same letter in a column are not significantly different from each other using the HSD test (P ≤ 0.05). Parameters: F_0 – minimal fluorescence (at t = 0); F_m, maximal fluorescence; F_v, maximum variable fluorescence; ϕP_0, maximum quantum yield of the primary PSII photochemistry; ΨE_0, probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A-$roba_0$, quantum yield for electron transport from Q_A-uantum yield for $elec_0$, probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side; ϕR_0, quantum yield for the reduction of the end electron acceptors at the PSI acceptor side; ϕD_0, quantum yield (at t = 0) of energy dissipation.

high concentrations of the metals (1 mM Cd and 5 mM Zn) suggest that *A. arenosa* is as tolerant to Cd and Zn excess as *A. halleri*, which was not reported so far (**Figure 4A**). In contrast, Paunov et al. (2018) reported a substantial decrease in chlorophyll content in leaves of durum wheat after 7 days treatment with only 50 μM Cd and 600 μM Zn. Küpper et al. (2007) also showed a significantly higher decrease in chlorophyll content in non-hyperaccumulator *Thlaspi fendleri* after exposure to Cd when compared with *Noccaea caerulescens*.

It was documented in the current study that flavonol and anthocyanin content was constitutively higher in *A. halleri* than in *A. arenosa* (**Figures 4B,C**). Generally, the content of flavonols lowered in both species after exposure to Cd or Zn, whereas the content of anthocyanins increased. The role of flavonols and anthocyanins during metallic stress has been poorly investigated

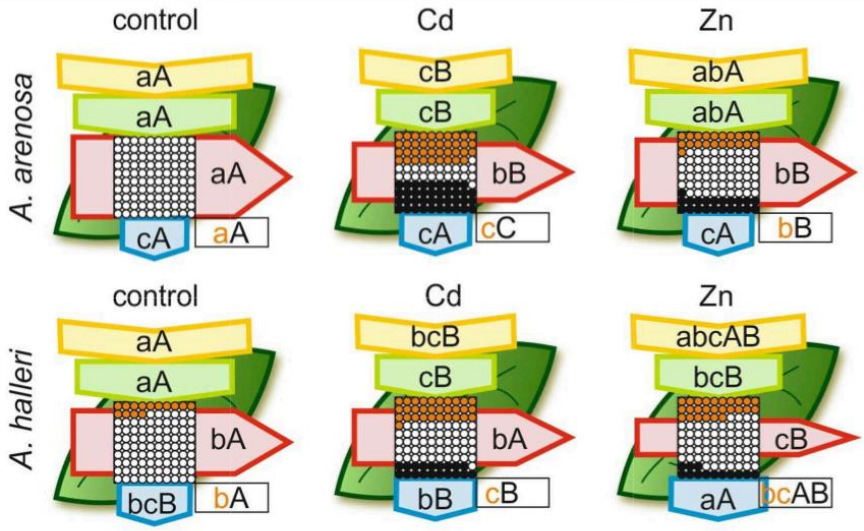

FIGURE 6 | Leaf model showing the phenomenological energy fluxes per the excited cross sections (CS) of the leaves of the *A. arenosa* and *A. halleri* at the end of experiment (120 h). Cd—1.0 mM Cd treatment; Zn—5.0 mM Zn treatment. Each relative value of the measured parameters is the mean ($n = 20$) and the width of each arrow corresponds to the intensity of the flux. Yellow arrow—ABS/CS, absorption flux per CS approximated; green arrow—TR/CS, trapped energy flux per CS; red arrow—ET/CS, electron transport flux per CS; blue arrow—DI/CS, dissipated energy flux per CS; circles inscribed in squares—RC/CS, % of active/inactive reaction centers. White circles inscribed in squares represent reduced Q_A reaction centers (active), black (or orange) circles represent non-reduced Q_A reaction centers (inactive), 100% of the active reaction centers responded with the highest mean value observed in the control conditions. Orange circles present the difference in % of inactive RC between *A. arenosa* and *A. halleri*. Means followed by the same letter for each parameter in a row (upper case letters) or in a column (lower case letters) are not significantly different from each other using the Tukey HSD test ($P < 0.05$). Letters are inscribed into arrows, except for RC/CS, where they are placed in a box in the bottom right corner of the square with circles. Lower case letters describe statistical differences between species in all treatments, upper case letters describe changes for each species during experiment. Orange lower case letters describe statistical differences in RC/CS between species under each HM treatment.

in plants so far. Nonetheless, Corso et al. (2018) showed recently that flavonols and anthocyanins play an important role in plant response to TME stress in hyperaccumulating and non-hyperaccumulating plant species, most likely through the enhancement of antioxidant capacity and/or metal chelation in plants (Corso et al., 2018). Our results may suggest that *A. arenosa* and *A. halleri* favor biosynthesis of anthocyanins over flavonols in response to the toxic effect of Cd or Zn (**Figures 4B,C**). However, it is possible that flavonols made the chelates with Cd and Zn and were not detected by the method used in the current study. Such lower detectability of flavonols by fluorometric methods, as a result of chelation with TME, was documented by Kasprzak et al. (2015). Hence, the role of flavonols in response to Cd and Zn toxicity in both plant species should be further investigated.

There is a dearth of data focusing on toxic effects of Cd and Zn on photosynthesis presented as chlorophyll *a* induction transients in metal hyperaccumulator and hypertolerant species such as *A. halleri*, moreover, there are no such reports for *A. arenosa*. Our data showed the presence of ΔK, ΔJ, ΔI, ΔH, and ΔG peaks on ΔV_t curves. Positive peaks on ΔV_t curves can be linked to the toxic effect of TME on different components of photosynthetic apparatus, whereas negative values might suggest stimulatory effect compared with the control. Under 1.0 mM Cd treatment in *A. arenosa*, which may suggest damage to the OEC (positive ΔK peak), electron transport between Q_A and Q_B (ΔJ) and connectivity between PSII and PSI, as well as the end electron acceptors of PSI like FNR (ΔI, ΔH, and ΔG) (**Figure 5A**) (Kalaji and Loboda, 2007; Paunov et al., 2018). On the other hand, in *A. halleri* that accumulated significantly less Cd (**Figure 1A**), we observed a stimulation of most of components of electron transport chain compared with the control (**Figure 5B**). Our research for the first time shows such stimulatory effect of Cd on photosynthetic apparatus of *A. halleri*. However, Tang et al. (2016) reported increase in plant growth as well as induction of some photosynthetic parameters (e.g., ϕP_0) and up-regulation of genes involved in photosynthesis in hyperaccumulator *Sedum alfredii* exposed to low Cd concentration (5 μM). Moreover, similar stimulation of growth of another hyperaccumulator *Noccea caerulescens* was reported by Lombi et al. (2000) that was exposed to Cd (100 μM). By contrast, Paunov et al. (2018) observed a presence of positive ΔK and ΔJ, and negative ΔI and ΔH peaks in ΔV_t curve for durum wheat plants treated with 50 μM of Cd for 7 days, whereas Kalaji and Loboda (2007)

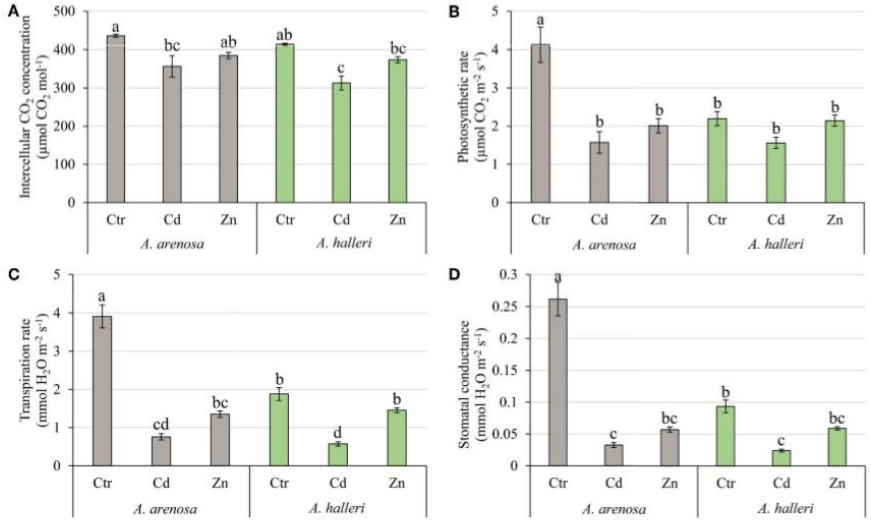

FIGURE 7 | Intercellular CO_2 concentration **(A)**, Photosynthetic rate **(B)**, Transpiration rate **(C)**, and Stomatal conductance **(D)** in *A. arenosa* (gray) and *A. halleri* (green) after 120 h of treatment. Ctr—control; Cd—1.0 mM Cd treatment; Zn—5.0 mM Zn treatment. Values are means ± SE ($n = 20$). Means followed by the same letter are not significantly different from each other using the HSD test ($P < 0.05$).

and Kalaji et al. (2018) reported the complete flattening of chlorophyll *a* fluorescence induction curve for barley treated with only 25 μM Cd for 24 h, which suggested lethal effect of investigated treatment.

Little information is available on the toxic effect of Zn on chlorophyll *a* fluorescence induction curves in plants and PSII functionality under Zn stress (Paunov et al., 2018; Moustakas et al., 2019). Paunov et al. (2018) showed the toxic effect of high Zn (600 μM) treatment, visible as a presence of positive ΔK and ΔJ peaks on chlorophyll *a* fluorescence induction transient in durum wheat. In current research we used 8-fold higher Zn concentration than that used by Paunov et al. (2018) and we showed that OEC (positive ΔK peak), Q_A pool (positive ΔJ peak) and PSI components (positive ΔI, ΔH, and ΔG peak) were more affected in *A. arenosa* (**Figure 5C**) compared with *A. halleri* (**Figure 5D**) despite higher Zn accumulation in leaves of *A. halleri* (**Figure 1B**). It should be stressed, however, that the Zn concertation used in the present study was 8-fold higher compared to the concentration used by Paunov et al. (2018).

Our results show that both Cd and Zn have contrasting effects on photosynthetic apparatus of *A. arenosa* and *A. halleri*. Based on presented chlorophyll *a* fluorescence induction curves and available literature we can conclude that photosynthetic apparatus of metallicolous *A. arenosa* population is hypertolerant to Cd and Zn stresses. Although Przedpełska and Wierzbicka (2007) reported that metallicolous population of *A. arenosa* are more tolerant to Cd and Zn than non-metallicolous populations, they did not compare them with any well-known hyperaccumulator plant of both metals.

Maximum quantum efficiency of the PSII (ϕP_0), otherwise known as F_v/F_m, is the most commonly used parameter which describes the state of photosynthetic apparatus, and many reports show its decrease in various plant species under Cd (Huang et al., 2017 Paunov et al., 2018; Xue et al., 2018) or Zn stress treatment (Vassilev et al., 2011; Santos et al., 2014; Paunov et al., 2018). Our results showed that Cd treatment caused ϕP_0 of *A. arenosa* and *A. halleri* to decrease near the end of experiment compared with the control plants, however, considering extremely high Cd concentration the overall physiological status of both species was not significantly reduced. Moreover, ψE_0 and ϕE_0 that are associated with O-J phase were not considerably affected by Cd treatment in both species (**Table 1**). In this report, exposure for 5 days to 5 mM Zn did not affect ϕP_0 of *A. arenosa*, whereas in *A. halleri* significant decrease was observed only at the end of experiment (**Table 1**). Other reports showing decreasing values of ϕP_0 in *A. arenosa*, *A. halleri* and *Noccea caerulescens* under Zn treatment (from 10 μM to 3.0 mM) are in good agreement with hypertolerance of photosynthetic apparatus of *A. arenosa* and *A. halleri* presented in the current study (**Table 1**) (Cho et al., 2003; Küpper et al., 2007; Przedpelska-Wasowicz and Wasowicz, 2013). Kalaji and Loboda (2007) reported that energy fluxes per excited cross section in two cultivars of *Hordeum vulgare* were significantly disrupted after 24 h treatment with 25 μM Cd. In their research in both cultivars, almost all RC were

deactivated by Cd and DI/CS was extremely higher compared to the control, causing ET/CS to be almost completely inhibited. The current study shows that 5 days treatment with 1 mM Cd or 5 mM Zn caused in both species that most energy fluxes per excited cross section, although significantly lowered compared with the control, had relatively high values and most of RC were still active (**Figure 6**). These results suggest that PSII of both species is hypertolerant to high concentration of Cd and Zn. For the first time, we show complete analysis of photosynthetic apparatus (**Table 1**) and phenomenological energy fluxes per excited cross section (**Figure 6**) for *A. arenosa* and *A. halleri* treated with high concentration of Cd and excess Zn in controlled conditions.

Our data showed that the studied metallicolous population of *A. arenosa* was extremely tolerant to Cd and Zn, similarly to the Zn, Cd hyperaccumulating *A. halleri* metallicolous population. This study also highlighted contrasting responses of plants growing at the same contaminated site to Cd and Zn treatments, in particular in metal accumulation, photosynthetic parameters [e.g., quantum yield (ϕP_0)], gas-exchange parameters (e.g., photosynthetic rate) as well as in the content of chlorophyll, flavonols, and anthocyanins.

AUTHOR CONTRIBUTIONS

MS, KS, NV, and EM conceived and designed the research. MS, KS, and ŻG conducted the experiments. MS, KS, SR, MC, and CH analyzed the data. MS wrote the first draft of the manuscript, which was edited by all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00748/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. CHAPTER III: Uptake, transport and detoxification of trace metal elements in pseudometallophytes: comparison of *A. halleri* and *A. arenosa*

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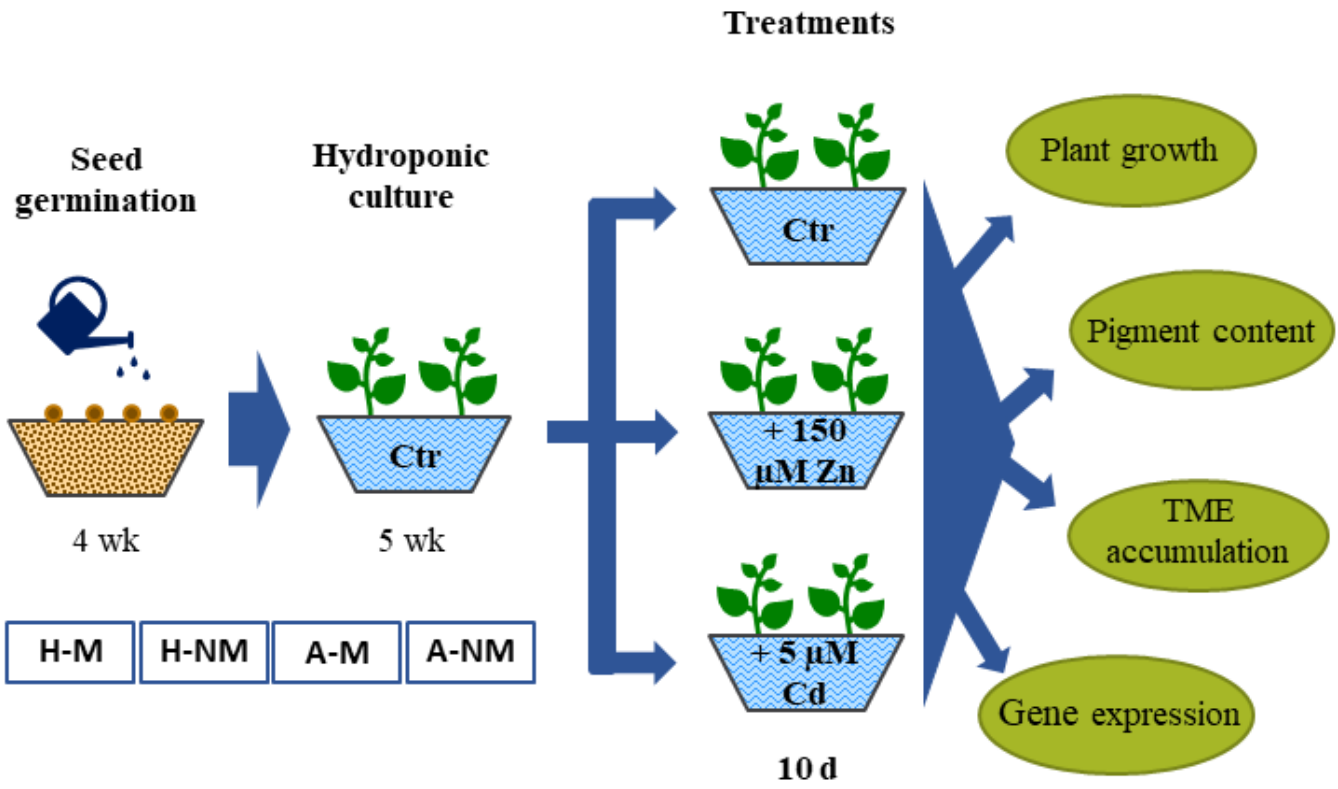

Graphical abstract

Uptake, transport and detoxification of trace metal elements in pseudometallophytes: comparison of *A. halleri* and *A. arenosa*

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Abstract

Trace metal elements (TMEs) such as Zn, Cu and Fe are essential in many aspects of plant growth and development but become toxic at high concentrations. On the other hand, Cd is an non-essential element that is toxic to plants even at low concentration. Pseudometallophyte plant species such as e.g. *Arabidopsis halleri* have long been used to study regulation of TMEs homeostasis and adaptation to metalliferous sites. In this study we performed comparative analysis of TMEs accumulation, pigment accumulation, level of Cd and Zn tolerance as well as expression level of genes involved in TMEs homeostasis between the *A. halleri* and *A. arenosa* populations from the same polluted site, together with the geographically close non-metallicolous (NM) populations of both species in control and under Zn and Cd treatment. Metallicolous (M) populations were more tolerant to Cd and Zn than NM populations with M population of *A. halleri* being more tolerant than *A. arenosa* M population. *A. halleri* M population was slightly more tolerant to Zn than M *A. arenosa*. Higher Cd and Zn accumulation was observed in shoots of *A. halleri* compared with *A. arenosa*. Higher flavonol content was observed in *A. arenosa* compared with *A. halleri*, which increased after Cd or Zn treatment. In

general the expression of many genes involved in metal homeostasis was higher in *A. halleri* than in *A. arenosa*. Triplication of HMA4 was confirmed in the two populations of *A. halleri*. Interestingly we found a triplication of *HMA4* in the *A. arenosa* M population. This triplication was not observed in the NM population. This suggests that *A. halleri* and *A. arenosa*, which grow at the same metalliferous site, show some degree of convergent evolution.

Keywords: Cadmium; Zinc; Metal hyperaccumulation; Metal transport; Metal detoxification

Introduction

Due to natural processes as well as industry and agriculture, environment becomes increasingly polluted with trace metal elements (TMEs), which poses a threat to public health (Kabata-Pendias & Pendias, 2001). Presence of toxic TME such as Cd and excess of essential TME Zn can have an adverse effects on plants. Despite the extensive study of interaction between TMEs and plants the mechanisms of uptake, translocation and detoxification of Cd and Zn are still largely uncovered (Aarts et al., 2012; Clemens, et al., 2013a; Balafrej et al., 2020; Corso & García De La Torre, 2020).

Arabidopsis halleri is a pseudometallophyte species often found on TME contaminated sites across Europe. Populations of *A. halleri* are characterized by high Cd and Zn accumulation and tolerance, with metallicolous populations being much more tolerant than non-metallicolous ones (Meyer et al., 2015; Sitko et al., 2017; Stein et al., 2017). The species show constitutive Zn tolerance and hyperaccumulation and Cd tolerance but high intraspecific variability in Cd accumulation ranging from hyperaccumulation to exclusion (Ge et al., 2000; Briskine et al., 2017; Corso et al., 2018, 2021; Babst-Kostecka et al., 2018). Phylogeographic studies suggest that metallicolous populations of *A. halleri* originated from nearby non-metallicolous populations that independently evolved the ability to colonize metalliferous sites (Moulon et al., 2005; Bonnin et al., 2006; Pauwels et al., 2012). As a well-known facultative hyperaccumulator of Cd and Zn, *A. halleri* has been widely used as a model species to study TME homeostasis and plant adaptation to metalliferous sites (Talke et al., 2006; Hanikenne et al., 2008; Verbruggen et al., 2009b, 2013a; Meyer et al., 2015; Corso et al., 2018). Many candidate genes underlying mechanisms of TME uptake, translocation and detoxification have been identified due to constitutively higher expression in *A. halleri* compared with its non-tolerant close relative model species *A. thaliana* (Krämer et al., 2007; Hanikenne et al., 2008; Verbruggen et al., 2009b, 2013b).

Arabidopsis arenosa is also a pseudometallophyte and close relative of *A. thaliana* species that can be commonly found growing alongside *A. halleri* on both metalliferous and non-metalliferous sites throughout Europe (Szarek-Lukaszewska & Grodzińska, 2011; Preite et al., 2019). Ploidy is variable in the species and most of the research on *A. arenosa* has focused on the phenomenon of polyploidization and adaptation of plants to genome duplication (Yant et al., 2013; Arnold et al., 2015; Kolář et al., 2016). However, the close relationship with both

A. halleri and *A. thaliana* and its adaptation to metalliferous environment makes *A. arenosa* a perfect candidate for comparative studies on metal homeostasis and the adaptation to extreme environments (Preite et al., 2019; Szopiński et al., 2019). So far, few metallicolous populations of *A. arenosa* were reported to exhibit very high tolerance to TMEs (Nadgórska-Socha et al., 2013; Przedpelska-Wasowicz & Wasowicz, 2013; Szopiński et al., 2019). Recently Szopiński et al. (2020) reported that metallicolous *A. arenosa* population exhibits Cd and Zn hyperaccumulation according to the definition by van der Ent et al. (2013).

Uptake, transport and detoxification of Zn and Cd in plants have been under investigation for a very long time. Nonetheless the full extent of mechanisms underlying Zn and Cd accumulation and tolerance have still not been fully revealed. Cd compete with other divalent ions like Zn, Fe and Mn for non-specific transporters (Krämer et al., 2007; Verbruggen et al., 2009a; Schvartzman et al., 2018). Investigations on hyperaccumulator and related non-hyperaccumulator plant species have shown that different regulation and constitutive expression of genes encoding transmembrane transporters is a major cause of contrasting TME accumulation strategies. Among the transporter families with a key role in hyperaccumulation phenomenon are the ZIP (Zinc-regulated transporter, Iron-regulated transporter protein), NRAMP (Natural Resistance Associated Macrophage), MATE (Multidrug and Toxin Efflux), YSL (Yellow-Stripe-Like), CDF (Cation Diffusion Facilitator), CAX (Cation Exchanger) and HMA (Heavy Metal Transporting ATPases) (Krämer et al., 2007; Rascio & Navari-Izzo, 2011; Baliardini et al., 2015; Balafrej et al., 2020).

IRT1 is a non-specific metal transporter from ZIP family responsible, together with the iron-reductase FRO2, for the uptake of Fe from the soil. It is highly induced by Fe deficiency (Connolly et al., 2002). IRT1 is also responsible for Cd, Zn and Mn uptake in *A. thaliana* (Lin et al., 2009; Shanmugam et al., 2011; He et al., 2017). In contrast to IRT1, another member of ZIP family, IRT3 is mainly involved in Zn uptake with low affinity for Fe in *A. halleri* (Lin et al., 2009). NRAMP3 and NRAMP4 are transporters located at the tonoplast, where they play an important role in metal ion homeostasis by the re-mobilisation of Fe and Mn from the vacuole in *A. thaliana* and *N. caerulescens*. Moreover it was speculated that NRAMP3 and NRAMP4 can also be involved in Zn, Cd, Cu, Co and Ni remobilisation (Van de Mortel et al., 2006; Oomen et al., 2009; Bastow et al., 2018). HMA2 and HMA4 are P1B-type pumps involved in Zn and Cd efflux from the cell and translocation from roots to shoots by loading these TMEs into the xylem (Kum et al., 2009; Hanikenne & Nouet, 2011). In particular, high

expression of *HMA4*, due to triplication and strong promoter activity, is considered as a main driving force behind Zn and Cd hyperaccumulation in *A. halleri* (Courbot et al., 2007; Hanikenne et al., 2008; Hanikenne & Nouet, 2011; Park & Ahn, 2014; Nouet et al., 2015). OPT3 is a plasma membrane oligopeptide transporter involved in root to shoot transition of Fe and was also proposed to be involved in Zn and Cd transport in *A. thaliana* (Mendoza-Cózatl et al., 2014; Zhai et al., 2014). Member of MATE family, FRD3 is a plasma membrane transporter located in the root pericycle involved in loading of citrate into the xylem. It plays an important role in Fe and Zn homeostasis, and was also suggested to be involved in translocation of other metals such as Cd in *A. thaliana* (Charlier et al., 2015; Zhou et al., 2019; Scheepers et al., 2020). Nicotianamine (NA) is a metal chelator playing an important role in Fe, Cu and Zn homeostasis (Callahan et al., 2008). Nicotianamine synthase (NAS) is the enzyme responsible for synthesis of NA, whereas ZIF1 is a transporter localised at the tonoplast to sequestrate NA inside the vacuole and was reported to be important for Zn tolerance in *A. thaliana* (Deinlein et al., 2012; Clemens et al., 2013a; Haydon et al., 2012). HMA3 and MTP1 are metal transporters responsible for metal sequestration inside the vacuole. While MTP1 is associated mainly with tolerance to Zn, HMA3 can transport wider range of metals in *A. halleri* (Fasani et al., 2017).

Comparisons between Cd hyperaccumulating and non-hyperaccumulating *A. halleri* populations also revealed that biosynthesis of flavonoids such as flavonols and anthocyanins is an important element in detoxification and tolerance mechanisms. Flavonoids are a large group of specialised metabolites (around 8000 metabolites) in plants. These phenolic compounds are characterised by two benzene rings linked together by heterocyclic pyran ring and they mainly occur as O-glycosides in plants (Winkel-Shirley, 2002; Tohge et al., 2017; Corso et al., 2020). F3H (Flavanone 3-hydroylase) and LDOX (Leucoanthocyanidin dioxygenase) are involved in later stages of flavonol and anthocyanin biosynthesis respectively (Corso et al., 2018). Flavonoids play an important role in protection of plants against large variety of negative effects caused by abiotic (e.g. excess of TMEs, UV-light and drought) as well as biotic (pathogens, herbivores and other plant competition) factors (Winkel-Shirley, 2002; Corso & García De La Torre, 2020; Corso et al., 2020). The profile of flavonoids accumulation can depend on adaptation of plants to their native sites (Winkel-Shirley, 2002; Corso et al., 2018, 2020; Szopiński et al., 2019; Corso & García De La Torre, 2020).

The present work compares physiology and expression of genes involved in metal homeostasis in *A. arenosa* and *A. halleri* populations that are growing on the same metallicolous and non-metallicolous sites in Southern Poland. Our results highlighted differences in the physiology and in the adaptation of the two pseudometallophytes. We observed a contrasting expression of genes involved in metal homeostasis and tolerance between *A. halleri* and *A. arenosa*. Most importantly triplication of *HMA4* gene was found in metallicolous population of *A. arenosa*.

Materials and Methods

Plant material and growth conditions

Seeds of metallicolous populations of *Arabidopsis halleri* and *Arabidopsis arenosa* were collected from the same metalliferous site in Piekary Śląskie and are referred to in this paper as H-M and A-M respectively. Seeds of non-metallicolous population of *A. halleri* were collected from site in Niepołomice and in this paper it is referred to as H-NM. H-NM population refers to PL13 population used by Meyer et al., 2010 (Meyer et al., 2010). Non-metallicolous population of *A. arenosa* seeds were collected from site near Klucze and in this paper it is referred to as A-NM (Table S1). The populations used are the same the ones used by (Szopiński et al., 2020). Szopiński et al. 2020 showed that both H-M and H-NM populations are diploid, whereas A-M and A-NM populations are tetraploid.

H-M, H-NM, A-M and A-NM seeds were sown on vermiculite in controlled growth chamber (16h light d^{-1}; 100 µmol photons m^{-2} s^{-1} irradiance; 20°C : 18°C, day : night; 70% humidity). After 4 wk of growth, plants were transferred to 4L hydroponic containers filled with modified MS medium (K_2SO_4 (0.88 mM), KH_2PO_4 (0.25 mM), NaCl (10 µM), $CaCl_2$ (2 mM), $MgNO_4$ (1 mM), FeEDDHA (20 µM), H_3BO_3 (10 µM), $ZnSO_4$ (10 µM), $MnSO_4$ (0.6 µM), $CuSO_4$ (0.1 µM), and $(NH_4)_6Mo_7O_{24}$ (0.01 µM)) (Corso et al., 2018). Plants of each population were randomly distributed in containers, which were moved in the growth chamber randomly every week during change of the growth medium. After 5 wk of growth, plants from each population were separated into 3 treatment groups: control, 5 µM $CdSO_4$ treatment and 150 µM $ZnSO_4$. Treatment lasted for 10 d and each group consisted of 3 separate containers, number of plants in each container was: 21-23 for H-M, 12-15 for H-NM, 29-31 for A-M and 31-33 for A-NM.

After the 10 d of treatment, three biological replicates (pools of all individuals per population/growth condition) were harvested. Root tissues were separated from shoots and blotted dry, and each tissue was quickly weighed. For each sample, half of the material was flash frozen in liquid nitrogen and stored in -80°C. The other half was dried at 80°C for 72 h and used for mineral analysis.

Mineral analysis

Approximately 150 mg of dry plant material was digested in a microwave-assisted wet digestion system (ETHOS 1, Milestone, Italy) according to the procedure provided by the manufacturer (concentrated HNO_3 and 30% H_2O_2, 4:1 v/v). The mineral content in shoots and roots were analysed in the digests using flame atomic absorption spectrophotometer (iCE 3500 FAAS, Thermo Scientific, USA). Reference plant material (Oriental Basma Tobacco Leaves (INCT-OBTL-5), Institute of Nuclear Chemistry and Technology, Poland) was used for the quality assurance of the analytical data.

Pigments analysis

The measurements of chlorophyll, flavonol and anthocyanin content indices were taken on fully developed leaves using a Dualex Scientific+ sensor (Force-A, France) without damaging the plants.

Total flavonoids were extracted from 100 mg of frozen plant material using 2 ml of methanol. The flavonoid content (Quercetin) was determined according to spectrophotometric method based on the formation of aluminium-flavonoid complexes (de Castilho et al., 2018). An aliquant of $AlCl_3$ solution (0.5 mL, 2 %, w/v) was added to 1 mL of the test solution (standard or sample) and subsequently 0.5 mL of water, 1M HCl was added. The mixture was vigorously shaken and then after 10 min of incubation at room temperature, subjected to spectral analysis at 425 nm. The amount of $AlCl_3$ solution was substituted by the same amount of water in blank. For quantitative analysis, quercetin (concentration range of 1–100 μg ml^{-1}) was chosen as the reference compound as it is widely found in plants. All analyses were carried out in 12 technical replicates using Microplate Reader (Molecular Devices, San Jose, USA).

Tolerance test

4 wk old plantlets of H-M (n = 23 for Cd and n = 20 for Zn), H-NM (n = 25 for Cd and n = 22 for Zn), A-M (n = 20 for Cd and n = 24 for Zn), A-NM (n = 17 for Cd and n = 24 for

Zn) and *A. thaliana* (col-0; n = 19 for Cd and n = 24 for Zn) were transferred from vermiculite into hydroponic containers filled with modified MS medium and randomly distributed. In order to asses Cd and Zn tolerance, each week plants were sequentially transferred to new medium with increasing concentrations of $CdSO_4$ (0, 25, 50, 75, 100, 150, 250, 350, 500, 750 μM) or Zn SO_4 (10, 50, 100, 400, 800, 1600, 3200, 6400 μM) . Each week, the plants were weighed after having gently dried the roots. Tolerance was determined as the lowest concentration at which no increase in biomass was observed (Bert et al., 2003).

RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was extracted from 100 mg of ground-frozen root and shoots samples using the Maxwell® LEV Plant RNA Kit (Promega, Madison, WI, USA). RNA was quantified with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Loughborough, UK).

cDNA was synthesized and Real-time PCR was performed in 96-well plates with the PikoReal real time PCR system (Thermo Scientific, Loughborough, 519 UK). Each 10 μL reaction consisted of: 2.5 μL cDNA, 5 μL SYBR Green mastermix (Promega, Fitchburg, WI, USA), 2 μL H_20, and 0.5 μM of each primer. A total of three technical repeats were run per cDNA and primer pair combination. qPCR reaction thermal profile was: pre-incubation at 95°C for 3 min, 40 cycles at 95°C for 30 s, 60°C for 1 min (Baliardini et al., 2015). Relative transcript levels were calculated by normalization to geometric mean of *EF1α* and *SHR* expression as a constitutively expressed reference genes (Talke et al., 2006; Meyer et al., 2016). Primers used for qRT-PCR analysis are presented in Table S2. For each primer pair and population (H-M, H-NM, A-M and A-NM) amplicons were sequenced (Figure S1). The DNA sequences were aligned using MUSCLE with the default settings. The tree was created by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analyses were conducted in MEGA7 (Kumar et al., 2016).

***HMA4* gene copy number**

For gene copy number genomic DNA of *A. halleri* (H-M and H-NM), *A. arenosa* (A-M and A-NM) and *A. thaliana* (col-0) species was extracted using CTAB method. Single copy

Short Root (*SHR*) gene was used as internal control and total of 17 technical replicates were used per DNA and primer pair combination according to the protocol described above. Primers have been described previously for *HMA4* (Hanikenne et al., 2013) and *SHR* (Baliardini et al., 2015). Primers used for genomic copy number analysis are presented in Table S3.

Statistical analysis and heat-map creation

Results are shown as means ± SE. The statistically significant differences among mean values were determined using one-way ANOVA and post hoc LSD test ($P < 0.05$). The statistical analysis was performed using Statistica v.13.1 (Dell Inc., USA) software. Heat maps and hierarchical clustering analyses of gene expression data were carried out using the 'heatmap.2' function (GPLOTS R package). The values used for the heat maps were calculated as $\log_2$.

Results

Cd and Zn tolerance

In order to assess the level of tolerance to Cd and Zn of H-M, H-NM, A-M and A-NM populations, a step-wise assay was performed in hydroponic conditions and concentrations at which no growth was observed. Plants of metallicolous populations of *A. halleri* and *A. arenosa* had higher Cd tolerance compared with non-metallicolous ones (Fig. 1a). H-M showed the highest Cd tolerance, and A-NM was the least tolerant to Cd (Fig. 1a). Zn tolerance pattern followed the same trend (Fig 1b).

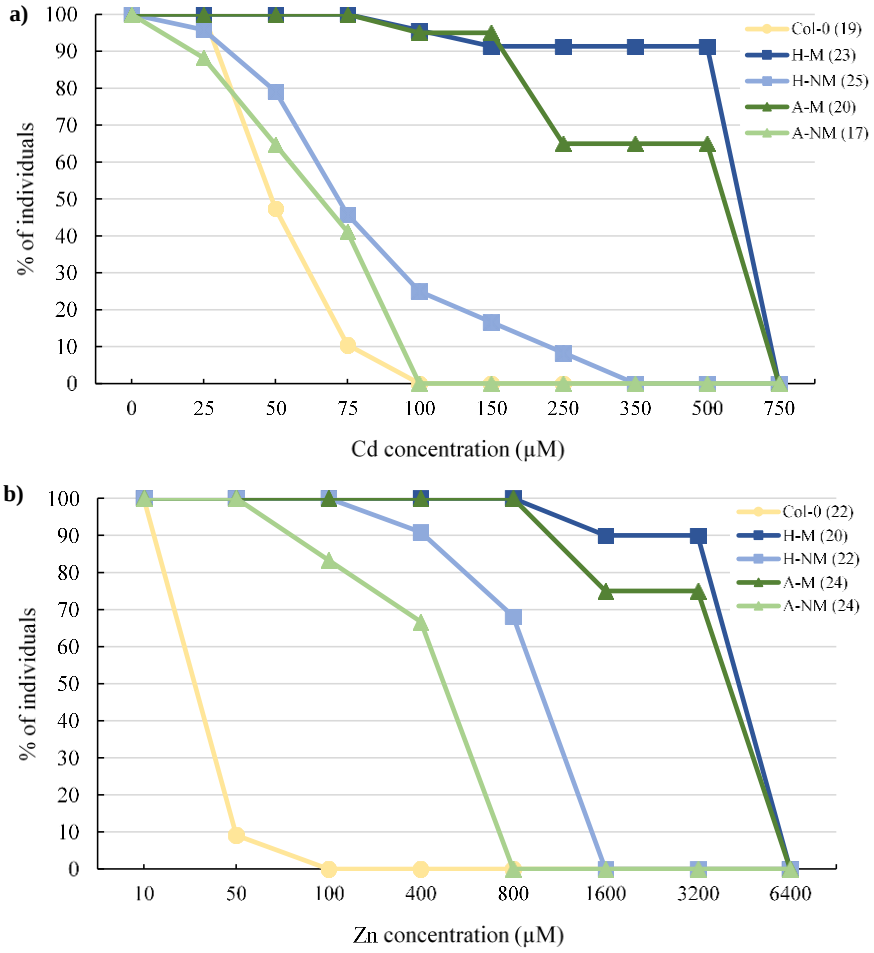

Figure 1. Distribution of Cd (a) and Zn (b) tolerance in the metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* as well as *Arabidopsis thaliana* ecotype Colombia (Col-0). Tolerance was assessed by exposing plants to increasing concentrations of Cd or Zn and determining the concentration at which no increase in fresh weight is observed.

The number of plants tested is indicated in the legend (n). The percentage of individuals from each tested population in each class is indicated, each class representing an interval of Cd and Zn concentrations (μM).

Fresh biomass of roots and shoots was measured in order to see impact of the Cd (5 μM) and Zn (150 μM) treatments on growth of individuals from investigated populations. In control conditions, roots of both *Arabidopsis halleri* populations had higher growth rates compared with *Arabidopsis arenosa* ones (Fig. 2a). No significant differences in shoot growth were observed between all investigated populations in control condition (Fig. 2b). After 10 days exposure to Cd (5 μM) or Zn (150 μM) only the growth rate of H-NM roots and shoots was decreased (Fig. 2a, b).

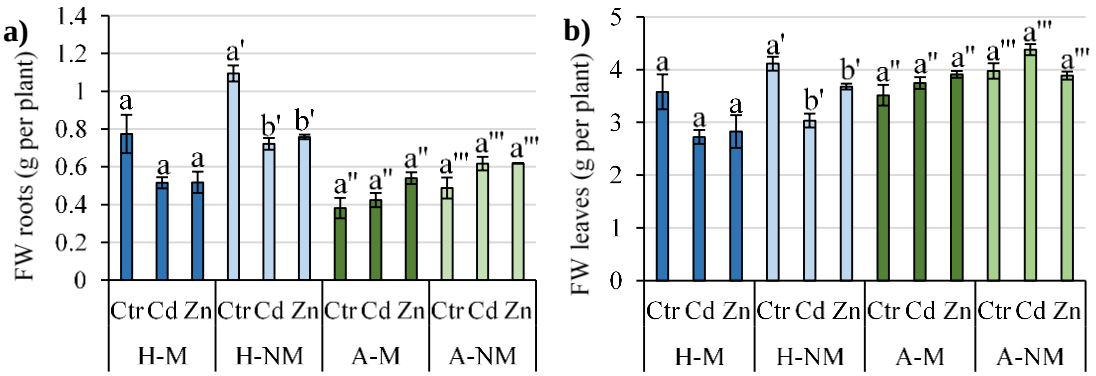

Figure 2. Fresh weight (FW) of roots (a) and shoots (b) per plant in metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 μM Cd (Cd) and 150 μM Zn treatment (Zn).

Metal accumulation in the roots and shoots

The concentrations of Cd, Zn, Fe, Cu, Mn and Mg in roots and shoots was analysed.

A-M and A-NM populations accumulated more Cd in roots compared with H-M and H-NM populations (Fig. 3a). In control all populations accumulated similar concentration of Zn in roots, moreover Cd treatment did not significantly affect Zn accumulation in roots (Fig. 3b). Under Zn treatment A-M population accumulated more Zn than H-M population, while there were no significant differences between Zn accumulation in roots of NM populations (Fig. 3b). Both M and NM *A. arenosa* population accumulated more Fe in roots compared with their *A. halleri* counterparts in control conditions (Fig. 3c). Cd and Zn treatment stimulated Fe accumulation in roots of H-M and H-NM populations, whereas in A-M and A-NM populations

Cd treatment lowered and Zn treatment did not significantly affected Fe accumulation in roots (Fig. 3c). Cd treatment significantly increased Cu accumulation in roots of H-M and H-NM populations, whereas both Cd and Zn treatment did not influence Cu accumulation in roots of A- and A-NM populations compared with control (Fig. 3d). A-M and A-NM accumulated significantly more Mn in roots in control compared with both *A. halleri* populations (Fig. 3e). Cd treatment significantly lowered accumulation of Mn in roots of all investigated populations compared with control, whereas Zn treatment did not cause a significant decrease in Mn accumulation compared with control only in H-M population (Fig. 3e). There was no observable trend in Mg accumulation in roots in all investigated populations (Fig. 3f).

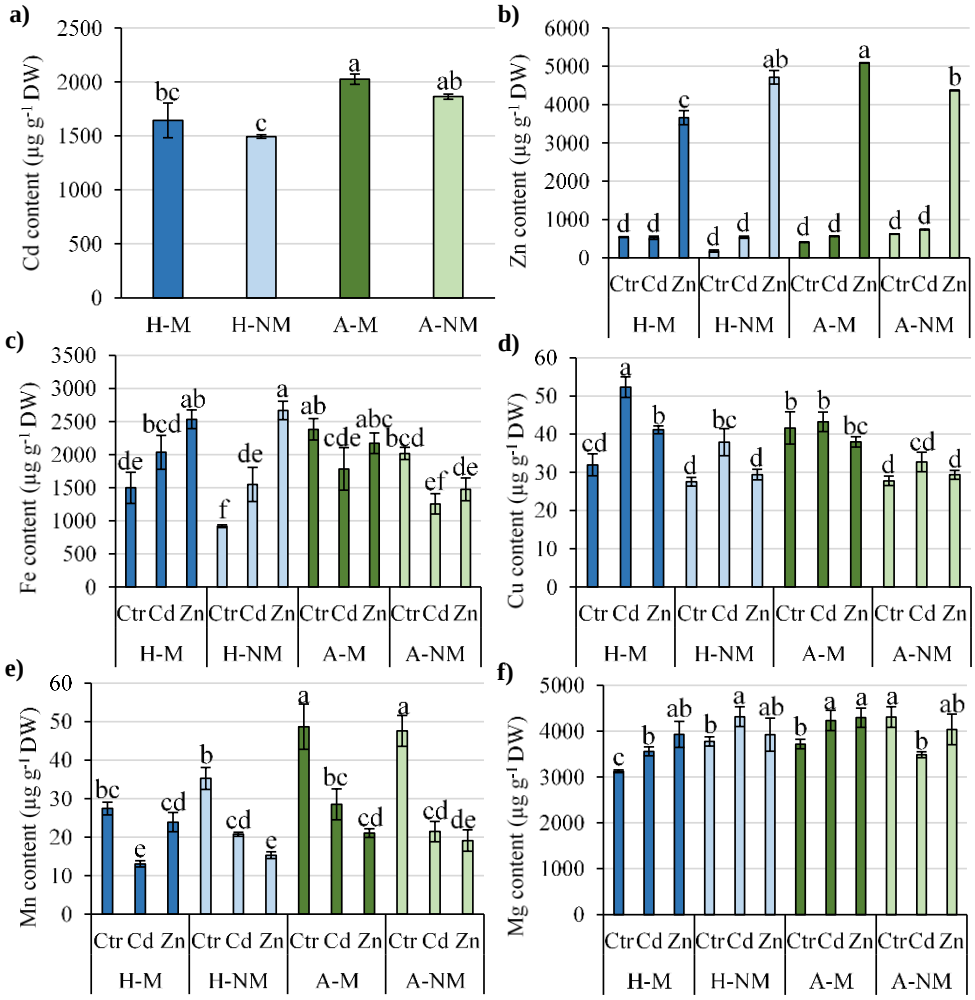

Figure 3. Accumulation of Cd (a), Zn (b), Fe (c), Cu (d), Mn (e), Mg (f) in roots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values are means ± SE (n= 3).

Shoots of both *A. halleri* populations accumulated more Cd and Zn than *A. arenosa* ones (Fig. 4a, b), H-NM showing the highest Cd and Zn accumulations. Fe concentrations were negatively impacted by Cd and Zn treatments in both NM populations, whereas in M populations only H-M showed lowered Fe concentration after Zn treatment (Fig. 4c). Cd and Zn treatments increased Cu concentrations in shoots of *Arenosa* plants, significantly in AM, in which the concentration was also the lowest in control condition (Fig. 4d). Mn concentration was decreased by Cd and Zn treatments only in H-NM plants, which also contained a much higher concentration in control condition (Fig. 4e). Mg concentration was increased compared with control only in *A. arenosa* shoots, in A-M population under Zn treatment and A-NM population under Cd treatment (Fig. 4f).

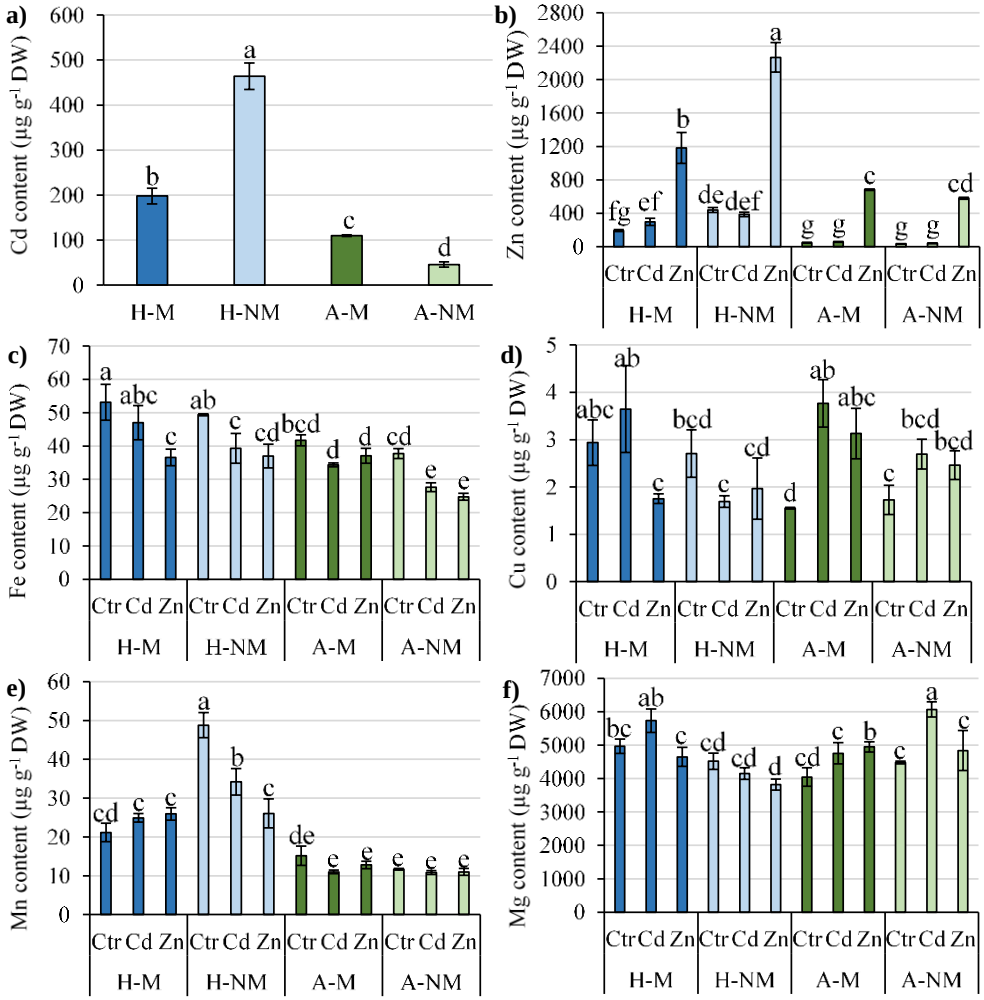

Figure 4. Accumulation of Cd (a), Zn (b), Fe (c), Cu (d), Mn (e), Mg (f) in shoots of metallicolous (H-M) and non- metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values are means ± SE (n= 3).

Chlorophyll, anthocyanin and flavonol content

Content of chlorophyll, anthocyanins and flavonols in leaves was measured in order to assess the overall state of plants in response to metal stress.

In control condition, A-NM population had lower chlorophyll content index, than the other populations (Fig. 5a). Chlorophyll content index decreased upon Cd treatment only in the two non-metallicolous populations but increased upon Zn treatment in the H-M population (Fig. 5a).

Anthocyanin content index was not modified by the treatments except in H-NM population treated with Zn (Fig. 5b).

Flavonol content index only increased in *A. arenosa* under Cd and Zn treatments compared with control conditions. In *A. halleri* populations the flavonol content index was constitutively higher in H-M population compared with H-NM (Fig. 5c). We also measured the content of quercetin, a well-known flavonol with antioxidant properties, previously found to accumulate in another Polish metallicolous population of *A. halleri* (Corso et al. 2018). H-M population showed constitutively higher Quercetin content compared with H-NM (Fig. 5b), with no change upon Cd and Zn treatment. On the other hand both Cd and Zn treatments caused a significant increase in Quercetin content in both *A. arenosa* populations (Fig. 5d).

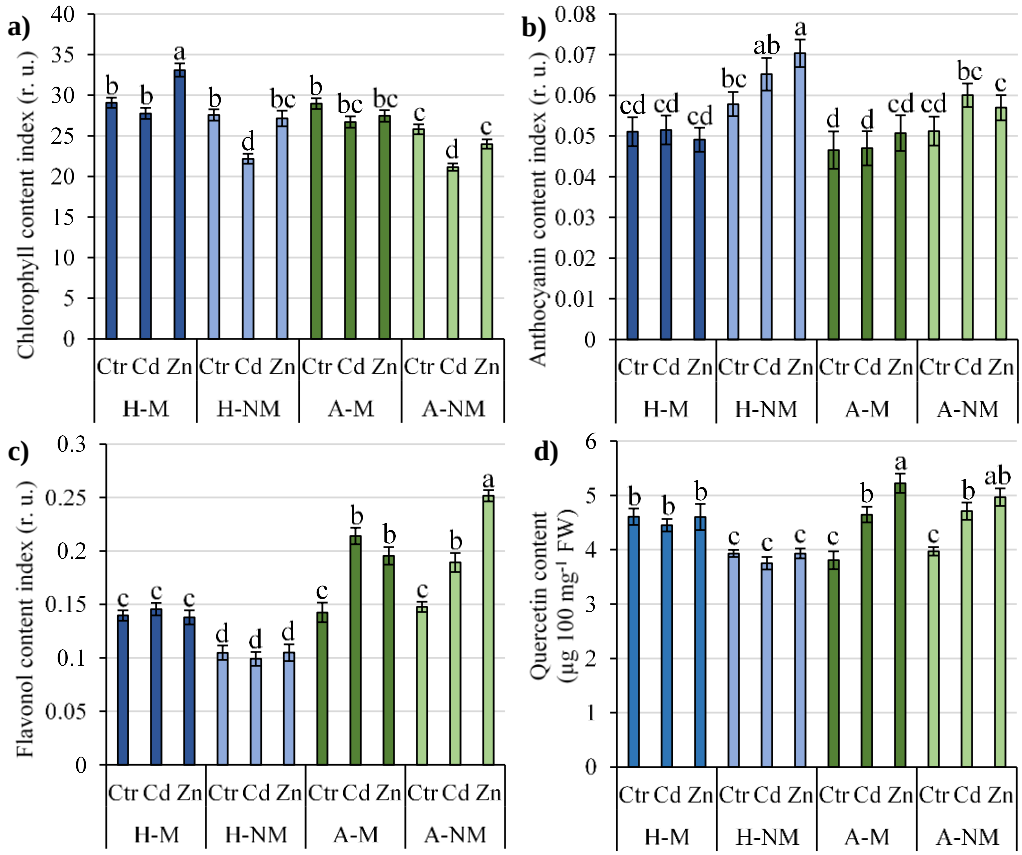

Figure 5. Chlorophyll (a), anthocyanin (b), flavonol (c) content index (mean ± SE; n= 30) and Quercetin content in leaves (mean ± SE; n= 12) (d) in metallicolous (H-M) and non- metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-M) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn).

Gene expression

The expression level of genes involved in the uptake, translocation and detoxification of TME, and previously shown to be differentially expressed in populations of *A. halleri* (Corso et al. 2018; Schvartzman, et al. 2018; Corso et al.2021), was investigated.

Genes involved in TME uptake

Expression of *IRT1* gene in roots was highly induced by both Cd and Zn in H-NM plants, while in H-M only Cd induced this gene to double compared with control. On the other hand in both *A. arenosa* populations only Zn treatment induced *IRT1* expression compared with control (Fig. 6a). Expression of *FRO2* in roots followed the same trend as IRT1 (Fig. 6b).

A-M showed much higher expression of *IRT3* in roots in control conditions compared to other populations. Both Cd and Zn treatments repressed *IRT3* expression in all populations compared to control, except in A-NM treated with Cd (Fig. 6c).

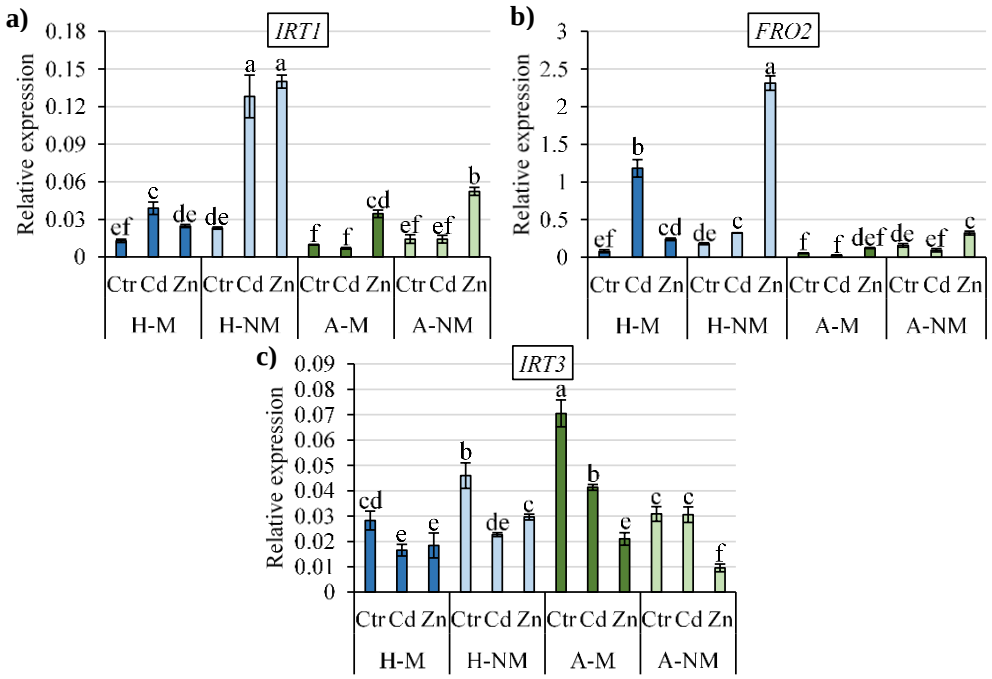

Figure 6. Relative expression of *IRT1* (a), *FRO2* (b), *IRT3* (c), in roots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values (mean ± SE; n=3 technical replicates) are given relative to the geometric mean of expression of reference genes *Elongation factor 1 alfa* and *Short Root*. Gene names are also reported.

Genes involved in TME transport and translocation

The highest level of *HMA2* expression in roots was found in A-M plants, moreover both Cd and Zn induced its expression compared to control conditions only in that population (Fig. 7a). In shoots *HMA2* expression was similar in all populations in control condition (Fig. 8a). Cd treatment caused a significant increase in *HMA2* expression in A-M and H-NM shoots , while similar increase was observed in Zn treated H-M and A-NM populations (Fig. 8a).

HMA4 expression also showed differences between populations. In roots, *A. halleri* NM and M populations showed opposite trend. In control conditions H-NM plants had around 3-times higher expression of *HMA4* compared with H-M and this expression was repressed by both Cd and Zn treatments. On the contrary, both Cd and Zn treatments induced the expression of *HMA4* in H-M. This induction was also observed, although to a lesser extent, in the A-M plants (Fig. 7b). On the other hand, in shoots, *HMA4* expression was highly induced by Cd treatment and not by Zn in all populations but to a lesser degree in the A-NM plants (Fig. 8b).

Both *A. halleri* populations were characterised by higher expression levels of *NRAMP3* in the roots compared with *A. arenosa* populations in control conditions (Fig. 7c). Under both Cd and Zn treatments the *NRAMP3* expression decreased in roots of H-M and H-NM plants. In contrast expression of *NRAMP4* was induced in A-M roots treated with Cd and Zn (Fig. 7c). In shoots, *NRAMP3* expression was not regulated by treatments in *A. halleri* plants and was clearly lower in A-NM in all conditions (Fig. 8c). The *NRAMP4* expression was significantly higher in roots of both NM populations compared with M ones in control conditions (Fig. 7d). Metal treatments induced expression in roots of all populations, except in A-NM in which it decreased. In shoots, metal treatments also induced expression of *NRAMP4* especially in *A. halleri* (Fig. 8d).

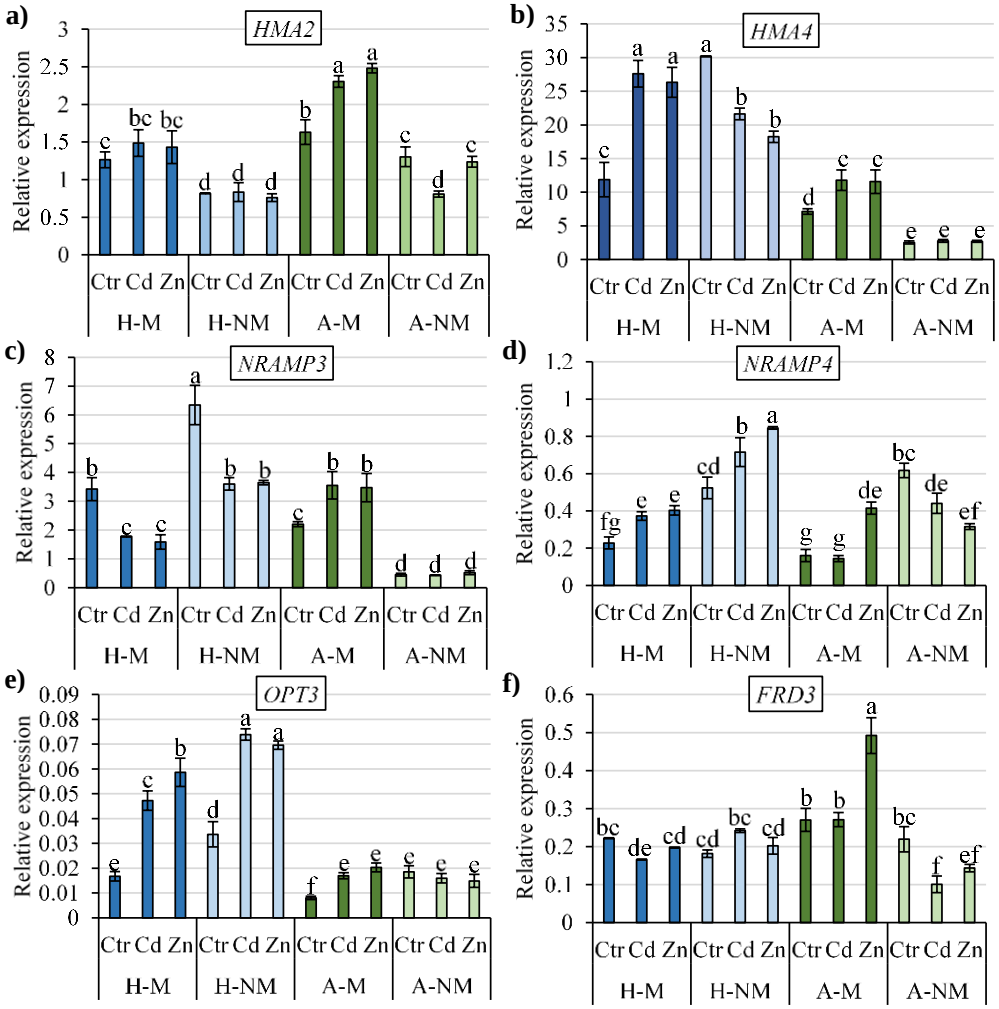

Figure 7. Relative expression level of *HMA2* (a), *HMA4* (b) *NRAMP3* (c), *NRAMP4* (d), *OPT3* (e) and *FRD3* (f), in roots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd)

and 150 µM Zn treatment (Zn). Values (mean ± SE; n=3 technical replicates) are given relative to the geometric mean of expression of reference genes *Elongation factor 1 alfa* and *Short Root*. Gene names are also reported.

Roots of both *A. halleri* populations showed much higher *OPT3* expression than *A. arenosa* ones. Cd and Zn treatments further increased this expression compared to control in *A. halleri.* Induction of *OPT3* was also observed to a much lesser extent in A-M roots (Fig. 7e). In shoots expression of *OPT3* was significantly induced by both Cd and Zn exposures in the NM populations but after different treatments in M populations: Cd induced expression in HM while Zn induced it in AM (Fig. 8e).

Expression of *FRD3* in roots was only affected by treatments in *A. arenosa*. In roots of A-M *FRD3* expression was doubled compared with control under Zn treatment, while in contrast Cd and Zn significantly decreased the expression of *FRD3* in A-NM roots compared with control (Fig. 7f).

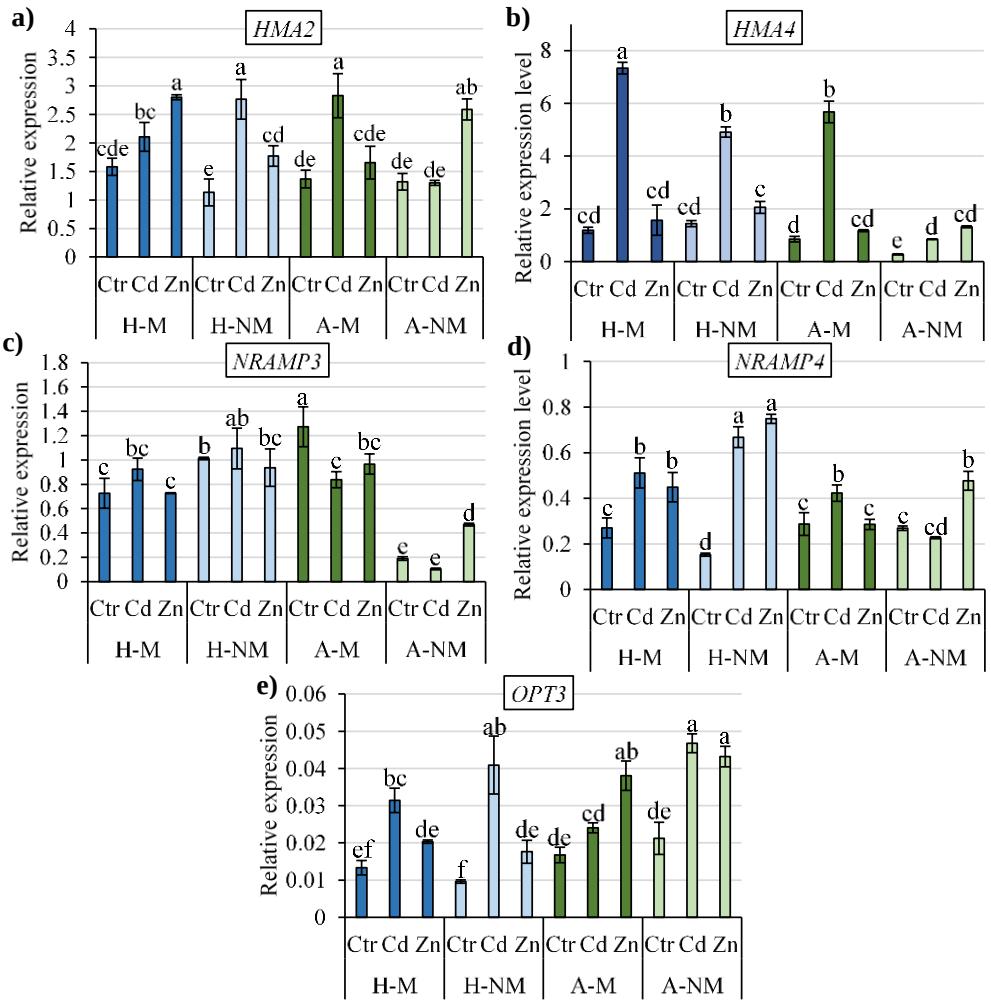

Figure 8. Relative expression level of *HMA2* (a), *HMA4* (b), *NRAMP3* (c), *NRAMP4* (d), *OPT3* (e), in shoots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values (mean ± SE; n=3 technical replicates) are given relative to the geometric mean of expression of reference genes *Elongation factor 1 alfa* and *Short Root*. Gene names are also reported.

Genes involved in TME detoxification

NAS2 expression was induced both Cd and Zn in all populations except A-NM. However the level of *NAS2* induction in *A. halleri* was double the one observed in A-M (Fig. 9a).

Expression profile of *ZIF1* in roots of both *A. halleri* populations followed similar expression profile as *NAS2* (Fig. 9b). In roots of both *A. arenosa* populations *ZIF1* expression was significantly increased only by Cd exposure compared with control (Fig. 10b). In shoots, *ZIF1* expression was particularly induced by both Cd and Zn treatments in the M populations. Zn had a bigger impact in H-M while Cd caused more significant induction of *ZIF1* expression in A-M (Fig. 10a).

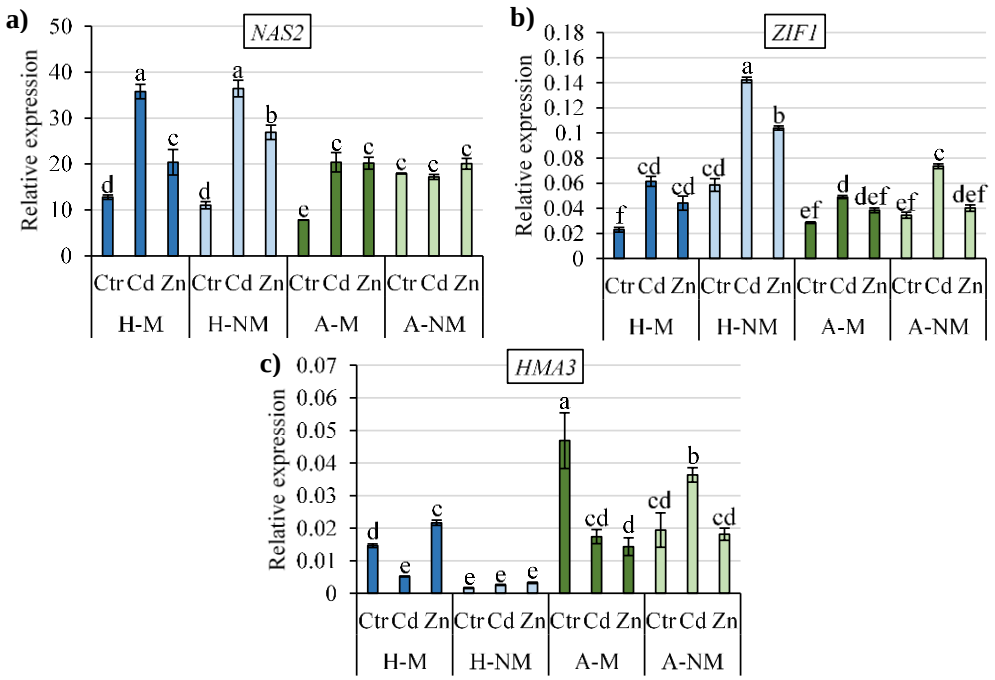

Figure 9. Relative expression level of *NAS2* (a), *ZIF1* (b), *HMA3* (c) in roots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values (mean ± SE; n=3 technical replicates) are given relative to the geometric mean of expression of reference genes *Elongation factor 1 alfa* and *Short Root*. Gene names are also reported.

In the roots, A-M population had much higher expression of *HMA3* in control conditions compared with other populations. Cd treatment lowered *HMA3* expression levels in both M populations, this trend was also observed for Zn treatment but only in A-M. On the contrary Cd induced *HMA3* in A-NM roots compared to control conditions (Fig. 9c). In marked contrast, shoots of both M populations showed induction of *HMA3*, with Zn having more impact on H-M and Cd on A-M. Interestingly the expression of *MTP1* was induced by Cd treatment and even more so in Zn treatment in H-M population, while on the other hand both treatments caused a decrease in expression in A-M population (Fig. 10c). Interestingly, expression of *HMA3* as well as *MTP1* was not affected by the treatments in shoots of both NM populations (Fig. 10b, c).

Cd and Zn strongly induced *F3H* expression in shoots of M populations, with Zn having more impact on H-M and Cd on A-M. (Fig. 10d). A milder induction was also observed in NM populations after Zn treatment. In the case of *LDOX* Zn treatment caused an 4-fold increase in expression in H-M shoots compared with other populations (Fig. 10e). To a lesser extent Cd treatment increased *LDOX* expression compared with control in both *A. arenosa* populations (Fig. 10e).

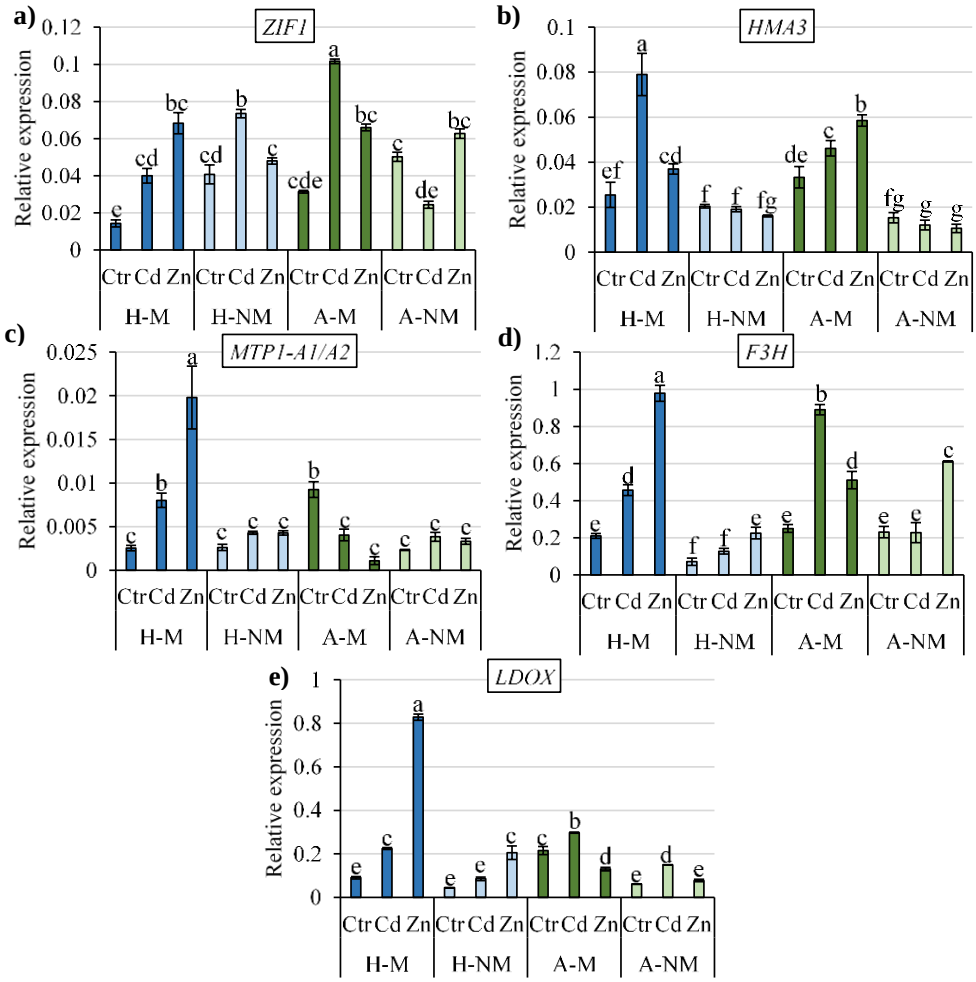

Figure 10. Relative expression level of *ZIF1* (a), *HMA3* (b), *MTPA1/A2* (c), *F3H* (d) and *LDOX* (e) in shoots of metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). Values (mean ± SE; n=3 technical replicates) are given relative to the geometric mean of expression of reference genes *Elongation factor 1 alfa* and *Short Root*. Gene names are also reported.

HMA4 gene copy number

The number of genomic copies of *HMA4* gene was analysed. *Arabidopsis thaliana* (Col-0) genomic DNA was used as the reference species with a single copy of *HMA4* gene. Single-copy *SHR* was used as a reference gene. Both *A. halleri* populations showed a triplication of *HMA4*. Triplication of *HMA4* was also observed in A-M population, whereas A-NM had only a single copy similarly to *A. thaliana* (Fig. 11).

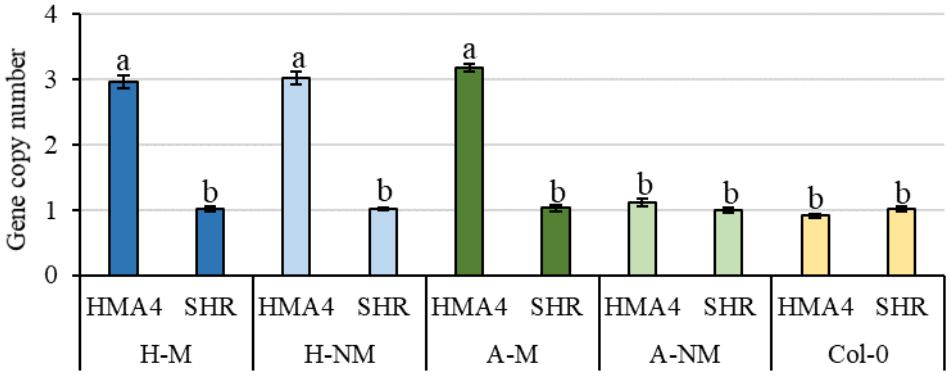

Figure 11. Gene copy number of *HMA4* in metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* populations. Values for *HMA4* copy number (mean ± SE; n=17 technical replicates) are given relative to the single-copy gene *Short Root* (*SHR*). *Arabidopsis thaliana* ecotype Colombia (Col-0) genomic DNA served as a calibrator.

Discussion

A. halleri and *A. arenosa* from metalliferous site show very high tolerance to Cd and Zn

Arabidopsis halleri as a well known hyperaccumulator of Cd and Zn have been widely used to study TMEs homeostasis in plants. Although a lot of progress has been made in discovering of metal transporters involved in the uptake, translocation and detoksyfication of Cd and Zn with the use of *A. halleri* as a model, the mechanism of TMEs homeostasis has not been fully explained (Verbruggen et al., 2009b; Balafrej et al., 2020; Corso & García De La Torre, 2020; Luo & Zhang, 2021). Study of metallophytes provides an unique oportunity to gain insight into the plant adaptation to metalliferous sites (Pollard et al., 2014; Corso & García De La Torre, 2020; Corso et al., 2021; Gieroń et al., 2021). Metallicolous population of *A. arenosa* from Piekary Śląskie (A-M) used in this study has been recently identified as a new hyperaccumulator of Cd and Zn by Szopiński et al. (2020). Moreover, Gieroń et al. (2021) reported that 5 out of 6 studied M populations of *A. arenosa* were hyperaccumulating Zn, while only 3 out of 6 were hyperaccumulating Cd. Such interspecies variability in hyperaccumulation capabilities observed for M populations of *A. arenosa* by Szopiński et al. (2020) and Gieroń et al. (2021) goes directly in hand with the same variability observed by Stein et. al. (2017) for *A. halleri*. Being closely related with *A. halleri*, *A. arenosa* being confirmed as hyperacumulator species will be a very usefull new model for study of plants adaptation to TMEs contamination and hyperaccumulation phenomenon. Studied M populations originate

from the same site highly contaminated with Zn and Cd and have been shown to be very tolerant to these TMEs both in the fileld and in the controlled conditions (Szopiński et al., 2020; Gieroń et al., 2021). We showed very high tolerance to Cd and Zn of *A. arenosa* M population (Fig. 1a, b) on the level comperable with the tolerance of *A. halleri* M populations reported to date (Bert et al., 2003; Meyer et al., 2015). Moreover, even both NM populations proved to be more tolerant to Zn and Cd compared to *A. thaliana* (Fig. 1a, b).

Flavonoids play a major role in tolerance to TMEs in *A. arenosa*

We observed that anthocyanin content was not affected by the treatments in H-M and A-M populations, while it was incresed by both Cd and Zn in H-NM and A-NM populations suggesting that used low concentration of Cd (5μM) and Zn (150μ) were not enough to induce the biosynthesis in M populations (Fig. 5b). On the other hand, flavonol content was increased by Cd and Zn treatment in *A. arenosa* irrespective of edaphic type compared with *A. halleri* populations in which treatments had no effect compared with the control (Fig.5c, d). Our findings suggest that flavonols play more important role in metal tolerance in *A. arenosa* than *A. halleri*. Similar results were observed by Szopiński et al. (2020), where Cd increased content of anthocyanins more in NM population of *A. arenosa* and *A. halleri*. Szopiński et al. (2020) also showed that Cd incresed flavonol content in *A. arenosa* NM and M populations compared with NM and M populations of *A. halleri*. Moreover, the results of Gieroń et al. (2021) showed higher content of flavonoids in *A. arenosa* populations adapted to metalliferous sites compared with NM populations. Importance of flavonoids in metal tolerance had been shown in *A. thaliana*, with mutants with genes involved in flavonoid biosynthesis knocked-out being much more sensitive to Cd compared with wild-type plants (Keilig & Ludwig-Müller, 2009). Very tolerant and hyperaccumulating populations of *A. halleri* have been shown to have high constitutive expression of genes related with flavonoid biosynthesis and higher accumulation of flavonoids compared with non-tolerant species (Schvartzman et al., 2018). Similarly, we also noted high expression of genes involved in flavonoid biosynthesis (*F3H* and *LDOX*) in both M populations (Fig.10 d, e), which further supports the importance of this group of specialised metabolites in the evolution of metal tolerance mechanisms. More in depth studies are needed in order to confirm if their role is mainly to scavenge reactive oxygen species (ROS) in order to protect against oxidative stress caused by TMEs (Mierziak et al., 2014; Shah & Smith, 2020)

or the formation of complexes with toxic metal ions to protect from their inhibitory effects and makes them easier to transport (Kasprzak et al., 2015; Corso et al., 2020).

Genetic analysis of *A. arenosa* and *A. halleri* supports some level of convergance between the two species

A. halleri and *Noccea caerulescens* species were used since the early days of hyperaccumulation studies, they show a large distribution in Europe and share high sequence conservation (94% and 88% respectively) with model species *A. thaliana*. Comparative genetic analysis using *A. halleri* and *N. caerulescens* with *A. thaliana* allowed the identification of many candidate genes involved in metal uptake, translocation and detoxification that were highly expressed in hyperaccumulating species compared with *A. thaliana* (Van De Mortel et al., 2006; Verbruggen et al., 2009b; Corso & García De La Torre, 2020). In recent years, the high intra-specific variability of accumulation and tolerance traits in *A. halleri* allowed to gain more insight into the molecular mechanisms of metal hyperaccumulation and hypertolerance (Stein et al., 2017; Corso et al., 2018, 2021; Schvartzman et al., 2018). However our current knowledge of intricacies of these mechanisms is still limited (Corso & García De La Torre, 2020; Luo & Zhang, 2021). The use of *A. arenosa* with the tools and resources already developed for closely related species *A. halleri* and *A. thaliana* might prove instrumental in gaining better understanding of the role of specific genes in TMEs homeostasis in plants or lead to identification of new ones. We observed that the expression of many genes involved in metal homeostasis was generally higher in *A. halleri* than in *A. arenosa*: *HMA4*, *NRAMP3*, *NRAMP4*, *OPT3*, *NAS2*, *ZIF1* (roots); *HMA4*, *NRAMP4*, *HMA3*, *MTP1*, *F3H*, *LDOX* (shoots) (Fig. 6-10). Higher expression of those genes in H-M population corelates with higher level of accumulation and tolerance to Zn and Cd. In particular *HMA4* expression level in roots was correlated with Cd accumulation in the four populations studied. The triplication of *HMA4* was observed in all populations of *A. halleri* explored so far irrespectively of the edaphic type of populations (Talke et al., 2006; Hanikenne et al., 2008; Hanikenne, 2011; Meyer et al., 2016; Baliardini et al., 2017). Interestingly the NM population of *A. halleri* showed much higher expression of *HMA4* in roots compared with M population (Fig. 7b), which may account for very high Cd and Zn accumulation in shoots observed by us and by Szopiński et al. (2020) both *in situ* and in hydroponic experiment. Our results show that this triplication of *HMA4* also occurred in *A. arenosa* at a later stage in the species history than *in A. halleri*, and was only observed in the

M population (Fig.11). This means that *Arabidopsis halleri* and *Arabidopsis arenosa*, which co-occur at the same calamine metalliferous site, show some degree of convergent evolution. These results are supported by Preite et al. (2019). Although the role of HMA4 in the shoots have not been well described, it may protect photosynthetically active tissues from TMEs damage (Corso & García De La Torre, 2020) by transporting excess of Cd and Zn out of the photosynthetic mesophyll cells. Expression of *HMA4* in shoots being highly stimulated by Cd (Fig. 8b) supports the high tolerance of H-M and A-M populations. Increased expression of *HMA3* in leaves has been correlated with Cd and Zn tolerance in plants (Ueno et al., 2011; Liu et al., 2017; Park & Ahn, 2017). Our results showed much higher expression of *HMA3* in shoots (Fig.10b) of A-M and H-M populations compared with NM populations, which suggests a major role in the high tolerance to Cd and Zn observed for these populations by Szopiński et al. (2019, 2020). Moreover, the differences in expression of *IRT1*, *FRO2*, *IRT3*, *NRAMP3*, *NRAMP4*, *FRD3* and *MTP1* (Fig.6-10) between H-M and A-M populations in response to Cd and Zn treatments suggest that the mechanisms of metal uptake, translocation and detoxification are at least partially different between the two species.

Conclusions

In this study we examined the differences in accumulation of TMEs, level of Cd and Zn tolerance and expression of genes involved in metal homeostasis in *A. halleri* and *A. arenosa* population from the same site heavily contaminated with metals and non-metallicolous populations from geographically close sites. Our data suggest that M population of *A. arenosa* and *A. halleri* display some degree of convergent evolution due to adaptation to the same metal contaminated environment. We observed that the *HMA4* triplication responsible for hyperaccumulation in *A. halleri* is also present in M population of *A. arenosa*. Observed differences in metal accumulation profile and expression of genes involved in metal uptake, translocation and detoxification in response to Cd and Zn treatment between *A. halleri* and *A. arenosa* M populations suggests that mechanisms of metal homeostasis are partially different between these two species. Based on our findings we suggest that *A. arenosa* species might be instrumental in the future studies to gain more insight in the regulation of mechanisms of metal accumulation and tolerance in plants.

Authors Contributions

Michał Szopiński, Nathalie Verbruggen and Eugeniusz Małkowski conceived and designed the research. Michał Szopiński, Krzysztof Sitko, Massimiliano Corso and Żaneta Gieroń conducted the experiments. Michał Szopiński, Massimiliano Corso and Nathalie Verbruggen analyzed the data. Michał Szopiński wrote the first draft of the manuscript, which was extensively edited by all of the authors.

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10. MOST IMPORTANT FINDINGS

This PhD project was aimed at improving the knowledge of the physiology of pseudometallophyte model species. In this work *A. halleri* and closely related pseudometallophyte species *A. arenosa,* were studied both *in situ* and in controlled hydroponic conditions. Metallicolous and non-metallicolous populations of *A. halleri* and *A. arenosa* species were chosen to assess the influence of edaphic type on the metal accumulation and

tolerance traits in both species. In order to answer the scientific questions put forward, a number of experiments were carried out. Results of these experiments were published in two papers Szopiński et al. (2020) – Chapter I, Szopiński et al. (2019) – Chapter II and the third is in preparation – Chapter III. Physiological parameters were measured in plants growing in their native sites, and the response of the four populations to Cd and Zn treatments was also studied in hydroponic conditions at low and high metal contaminations. Physiological analysis was complemented by molecular analysis. The expression of genes involved in Cd and Zn uptake, translocation and detoxification was compared between studied populations.

During the *in situ* investigation I observed that metallicolous population of *A. arenosa* accumulates Cd and Zn in leaves at a concentration far above hyperaccumulation thresholds (Chapter I). In *A. arenosa* hyperaccumulation was dependant on the edaphic type, which was not the case in the studied *A. halleri* populations. The *in situ* analysis of mineral composition, pigment content indices and activity of the photosynthetic apparatus showed distinct differences between the two species, which were even more pronounced in the laboratory experiments (Chapter I). These results showed that both species evolved partially different strategies for the adaptation to metalliferous site, which in turn provided the answer to the first scientific question put forward at the beginning of the project – *Does A. arenosa adapt the same way to contaminated environment as A. halleri in situ and in controlled conditions?* What was also of note is that the non-metallicolous population of *A. halleri* accumulated Cd and Zn at much higher levels compared that the neighbouring metallicolous population (Chapter I), with comparably lower tolerance levels to these metals (Chapter I and III). Based on the hypothesis that metallicolous populations of *A halleri* evolved from nearby non metallicolous ones, This suggests that mechanisms of transport limitation took place during the adaptation to the contaminated site.

My research shed light on the *A. arenosa* M population not only as a new hyperaccumulator species, but also as a first tetraploid hyperaccumulator species from Arabidopsis genus. However, my results showed that polyploidisation and Cd and Zn hyperaccumulation and tolerance traits are not necessarily linked in *A. arenosa* as the tetraploid NM population did not show these traits (Chapter I and III).

Although photosynthetic apparatus of both *A. halleri* and *A. arenosa* M populations proved to be very tolerant to Cd and Zn (Chapter I and II), we noted some differences between the species, especially in the quantum yield of photosynthesis, phenomenological energy fluxes

and photosynthetic rate. While exposed to increasing Cd concentrations or high concentrations of Zn, the photosynthetic apparatus of M *A arenosa* population, although highly resistant, was more susceptible to damage, compared with *A. halleri* M population (Chapter I and II). My findings suggest that *A. halleri* has more efficient mechanisms of Cd and Zn detoxification compared with *A. arenosa*, which is supported by higher expression levels of *HMA3* and *MTP1* genes responsible for Cd and Zn sequestration in vacuoles of leave cells (Chapter III).

Higher contents of flavonols and anthocyanins in M populations of *A. halleri* and *A. arenosa*, than in NM corresponding populations further support their importance in TME tolerance (Chapter I, II and III). Furthermore, contrasting accumulation of flavonols and anthocyanins between the two species under high Cd and Zn stress suggest differences in adaptation strategies of *A. halleri* and *A. arenosa* (Chapter I and II). Which was further supported by contrasting expression of genes involved in flavonoid biosynthesis, such as *F3H* and *LDOX* in response to Cd and Zn treatments in *A. halleri* and *A. arenosa* (Chapter III). However, further studies should be performed to specify the metabolites and to investigate the exact roles in the protection mechanisms, such as ROS scavengers or chelating agent.

During hydroponic experiments, I observed that under high Cd (1.0 mM) and Zn (5.0 mM) treatments *A. halleri* accumulates much more Zn in leaves, whereas *A. arenosa* accumulated much more Cd (Chapter II). Differences in physiological parameters, such as mineral composition, oxidative stress levels, activity of photosynthetic apparatus and the profile of pigments contents measured during this experiment provided and answer to the second scientific question – *Does A. arenosa metallicolous population respond the same way as A. halleri metallicolous population under short term treatment with high Cd and Zn concentrations?* I can clearly state that even though both studied populations adapted to the same metalliferous site, they evolved at least partially different mechanisms of metal homeostasis (Chapter I, II and III).

Despite *A. arenosa* and *A. halleri* being closely related species and the metallicolous populations investigated in this project grow on the same contaminated site, we observed many differences in the profile of mineral accumulation and other physiological parameters between them (Chapter I and II). Investigation of expression of selected genes involved in metal homeostasis (Chapter III) provided the answer to the third scientific question – *Are there differences in the mechanisms of metal homeostasis between A. halleri and A. arenosa. In particular can we observe the differences in the expression of genes involved in metal uptake,*

translocation and detoxification in metallicolous and non-metallicolous populations of A. halleri and A. arenosa in response to Cd and Zn treatments? Although differences in expression of genes involved in the TME uptake, translocation and detoxification between the two species were observed, some of the key findings underlying hyperaccumulation in *A. halleri* seemed to also be present in *A. arenosa*. Increased expression and most importantly the triplication of *HMA4* typically present in *A. halleri* species, was also found in *A. arenosa* M population. Although *HMA4* duplication is thought to have occurred at the speciation of *A. halleri*, my results suggest it at a later stage of the species history. A key aspect of tolerance mechanism in the two species seems to be also related to higher expression of *HMA3* (Chapter I and II) compared with NM populations allowing efficient detoxification of metals from the cytosol to the vacuoles (Chapter III). In short, my findings suggest some degree of convergent evolution between *A. arenosa* and *A. halleri* as a result of the adaptation to the same metalliferous site, however, mechanisms of TME homeostasis are at least differently regulated in these two species (Chapter III).

The results of this project present some interesting opportunities for future research. Addition of *A. arenosa* as a new hyperaccumulator species from genus Arabidopsis closely related with already established model species *A. halleri* and *A. thaliana*, opens up a new avenue of comparative molecular research. This research might help unravel the intricacies of molecular mechanism of metal homeostasis and their regulation in plants. My results suggest that the influence of genome duplication in *A. arenosa* species on metal tolerance and accumulation, should be further studied in the future research as a new interesting aspect previously omitted due to lack of polyploid hyperaccumulator species in Arabidopsis genus. More research comparing the differences between *A. arenosa* and *A. halleri* may bring us a step closer to making methods of cleaning up the contaminated environment with the use of plants such as phytoextraction and phytomining more viable in the future, by modifying plants with highly efficient accumulation mechanisms and increased tolerance. On the other hand better understanding the accumulation mechanisms can be used in biofortification research in order to construct plants with high concentrations of essential elements, while limiting the accumulation of toxic metals. Further, more in depth research in differences in activity of photosynthetic apparatus between *A. arenosa* and *A. halleri* may lead to development of fast and easy methods of detecting of metal stress in plants with the use of chlorophyll *a* fluorescence measurements. Moreover, deeper studies of the photosynthetic apparatus might be

very useful in elucidating the differences in mechanisms involved in protection of the photosynthetic apparatus in these species.

11. CONCLUSIONS

- *A. arenosa* from the metalliferous site is hypertolerant to Cd and Zn and hyperaccumulates these metals.
- The metallicolous population of *A. arenosa* is slightly less tolerant to Cd and Zn compared with the *A. halleri* population from the same site.
- *A. arenosa* from the metalliferous site is the first tetraploid hyperaccumulator species from Arabidopsis genus.
- Autotetraploidisation is not connected with hyperaccumulation and hyperaccumulation traits in *A. arenosa*
- Physiological parameters group the M populations closer together than the NM populations, which might suggest some degree of convergent evolution as adaptation to the same metalliferous site.
- *A. halleri* non-metallicolous population showed a highly efficient mechanism of Cd and Zn uptake and translocation into shoots compared to the metallicolous population. However, the tolerance of the *A. halleri* non-metallicolous population is much lower.
- Studied *A. arenosa* populations have a different profile of pigments accumulation compared to *A. halleri* populations. *A. arenosa* and *A. halleri* metallicolous populations show contrasting expression of genes involved in flavonol and anthocyanin biosynthesis.
- *HMA4* gene triplication in the *A. arenosa* metalliferous population and increased expression upon Cd and Zn treatment similarly to *A. halleri* might be further evidence of some degree of convergent evolution or gene transfer at earlier stages of evolution of this species.
- Contrasting expression of *IRT1* (uptake), *NRAMP3* and *NRAMP4* (remobilisation in roots), *HMA2* (translocation) as well as *MTP1* and *HMA3* (detoxification) between studied *A. arenosa* and *A. halleri* metallicolous populations suggests differences in mechanisms of TME homeostasis.

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13. SUPPLEMENTAL MATERIAL

13.1. Supplemental materials for Chapter I

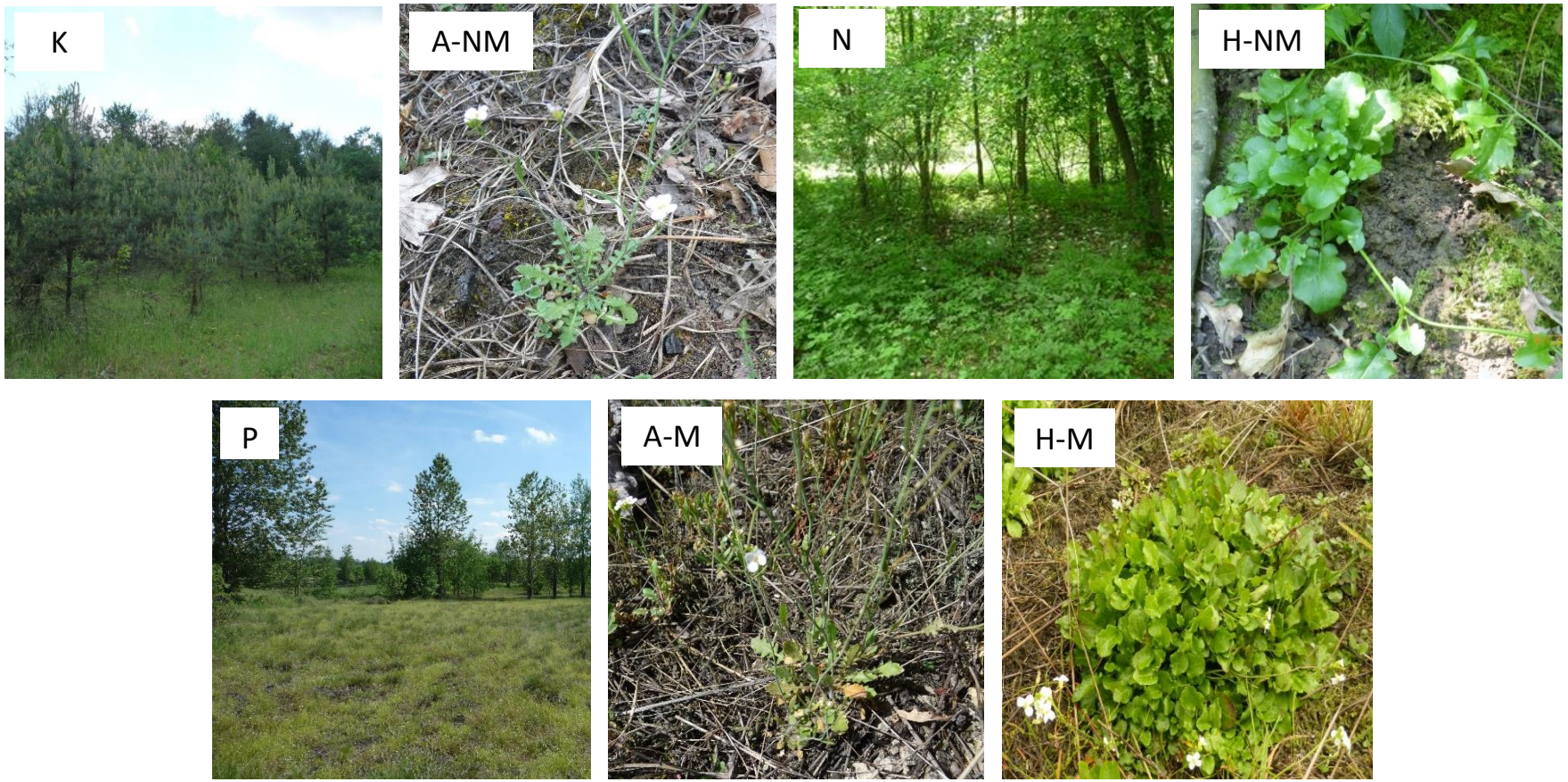

Figure S1. The pictures of investigated sites and typical plant from each species. Abbreviations of the site names: K – Klucze site (non-metallifereous) and A-NM – *A. arenosa* from the site; N – Niepołomice site (non-metallifereous) and H-NM – *A. halleri* from the site; P – Piekary Śląskie site (metallifereous); A-M – *A. arenosa* from Piekary Śląskie; H-M – *A. halleri* from Piekary Śląskie.

Figure S2. The pictures of siliques of *A. arenosa* (A) and *A. halleri* (B) from the metalliferous site in Piekary Śląskie.

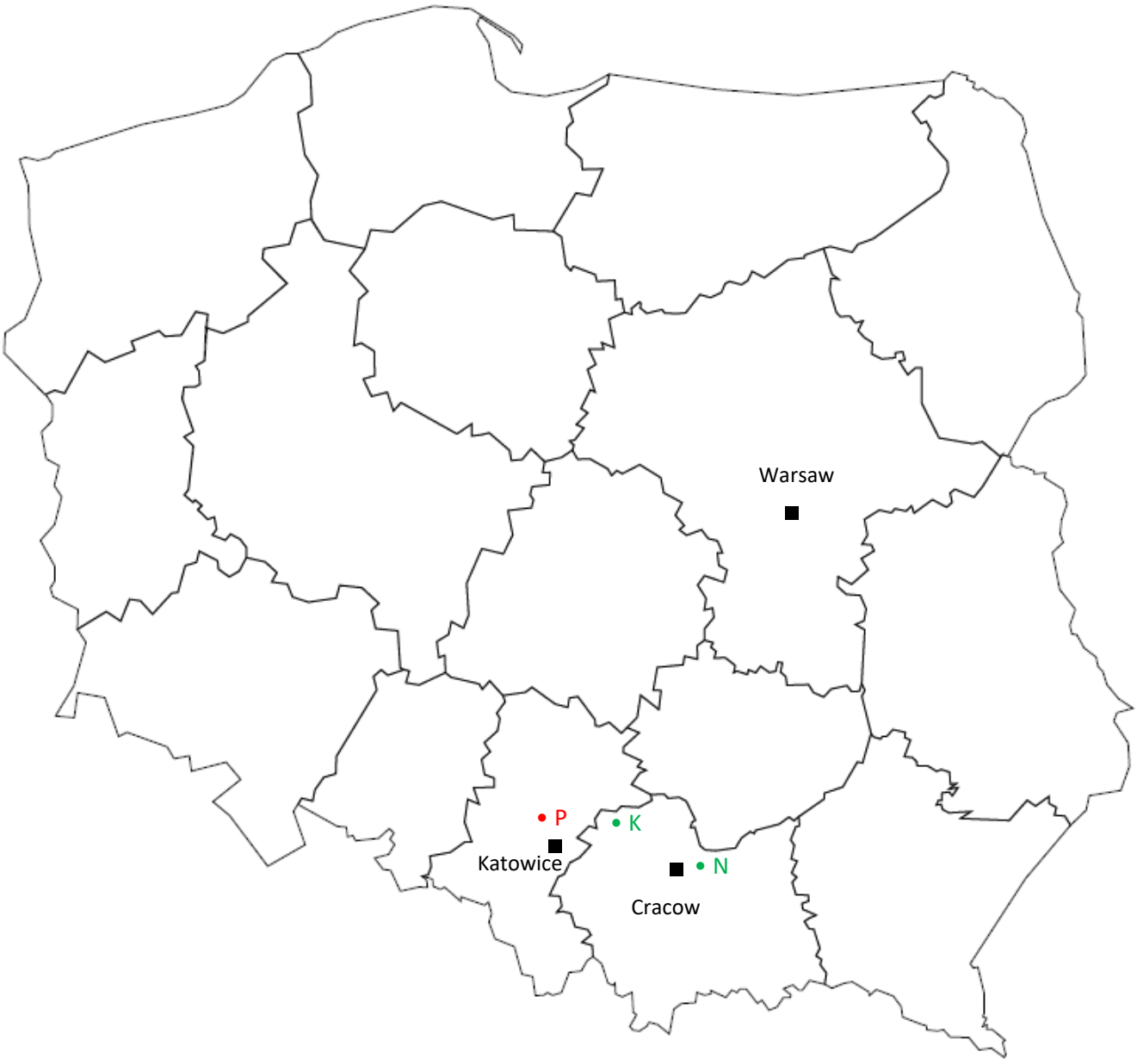

Figure S3. The overview map of location of investigated sites. The sites were marked as red (M populations) or green (NM populations) points. Abbreviations of the site names: P – Piekary Śląskie, K – Klucze, N – Niepołomice.

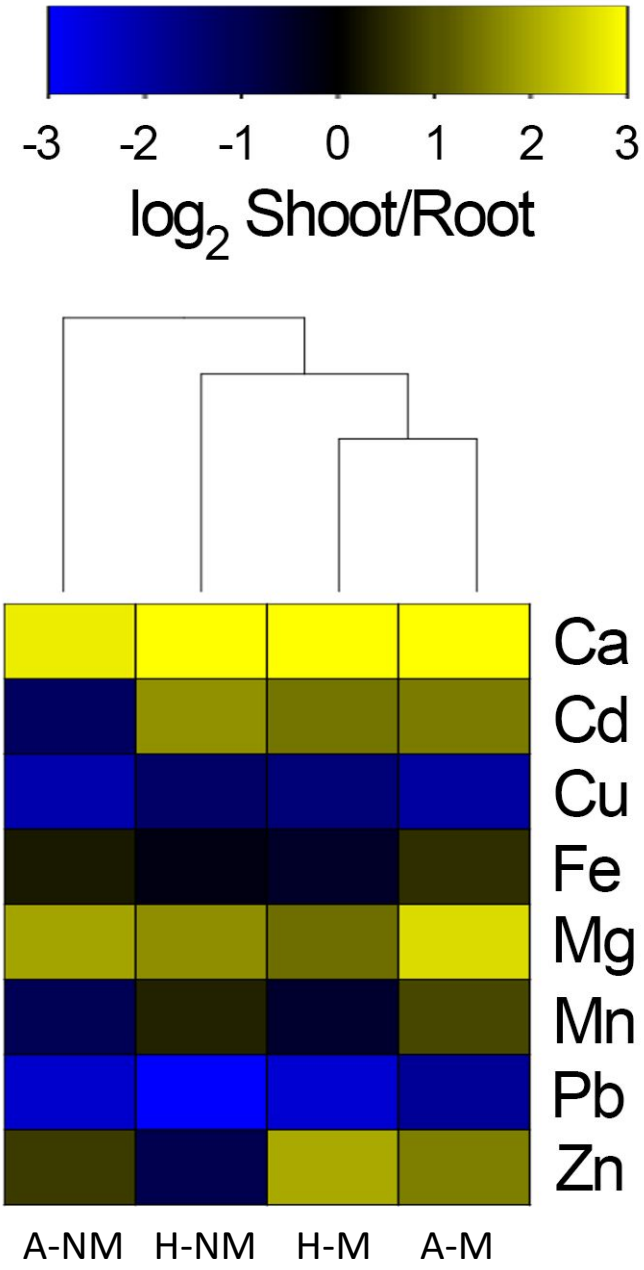

Figure S4. Heat map representing log_2 shoot : root ratio of essential mineral elements and cadmium concentrations in plants from A-NM, A-M, H-NM and H-M populations grown *in situ*. Yellow and blue indicate higher and lower mineral elements content, respectively, in shoot samples with respect to root.

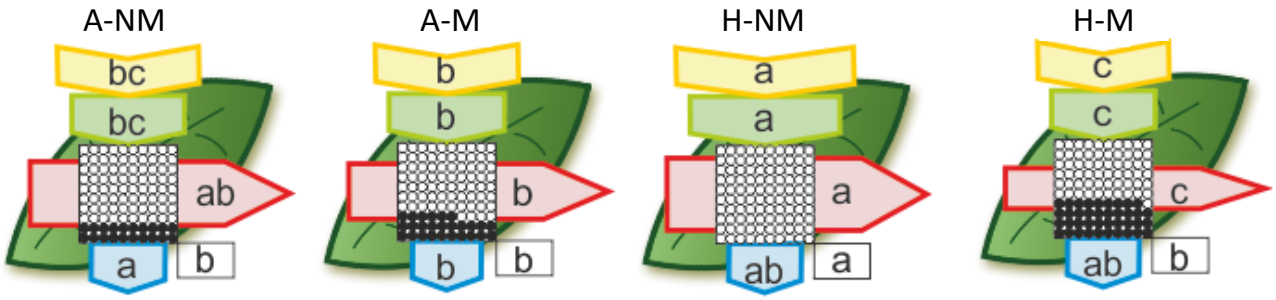

Figure S5. Leaf model showing the phenomenological energy fluxes per the excited cross sections (CS) of the leaves of the *A. arenosa* and *A. halleri* plants that were studied in field conditions. Each relative value of the measured parameters is the mean (n = 24) and the width of each arrow corresponds to the intensity of the flux. Yellow arrow – ABS/CS, absorption flux per CS approximated; green arrow – TR/CS, trapped energy flux per CS; red arrow – ET/CS, electron transport flux per CS; blue arrow – DI/CS, dissipated energy flux per CS; circles inscribed in squares – RC/CS, % of active/inactive reaction centers. White circles inscribed in squares represent reduced Q_A reaction centers (active), black circles represent non-reducing Q_A reaction centers (inactive), 100% of the active reaction centers responded with the highest mean value observed in H-NM population. Means followed by the same letter for each parameter are not significantly different from each other using the HSD test ($P < 0.05$). Letters are inscribed into arrows, except for RC/CS, where they are placed in a box in the bottom right corner of the square with circles. Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

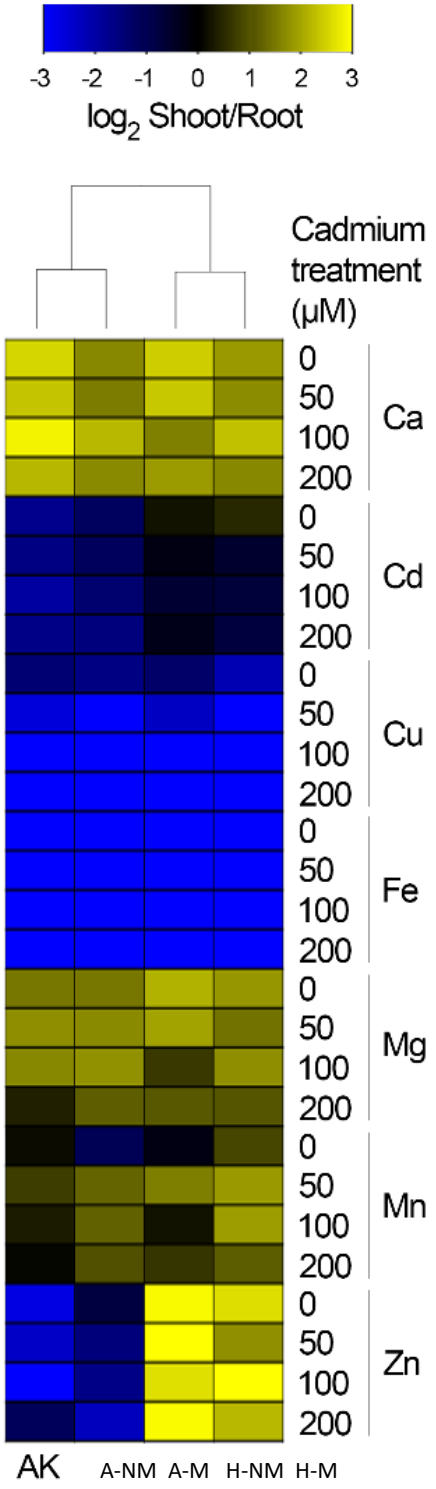

Figure S6. Heat map representing log_2 shoot : root ratio of essential mineral elements and cadmium concentrations in plants from A-NM, A-M, H-NM and H-M populations grown in hydroponic at 0, 50, 100 and 200 µM cadmium. Yellow and blue indicate higher and lower mineral elements content, respectively, in shoot samples with respect to root.

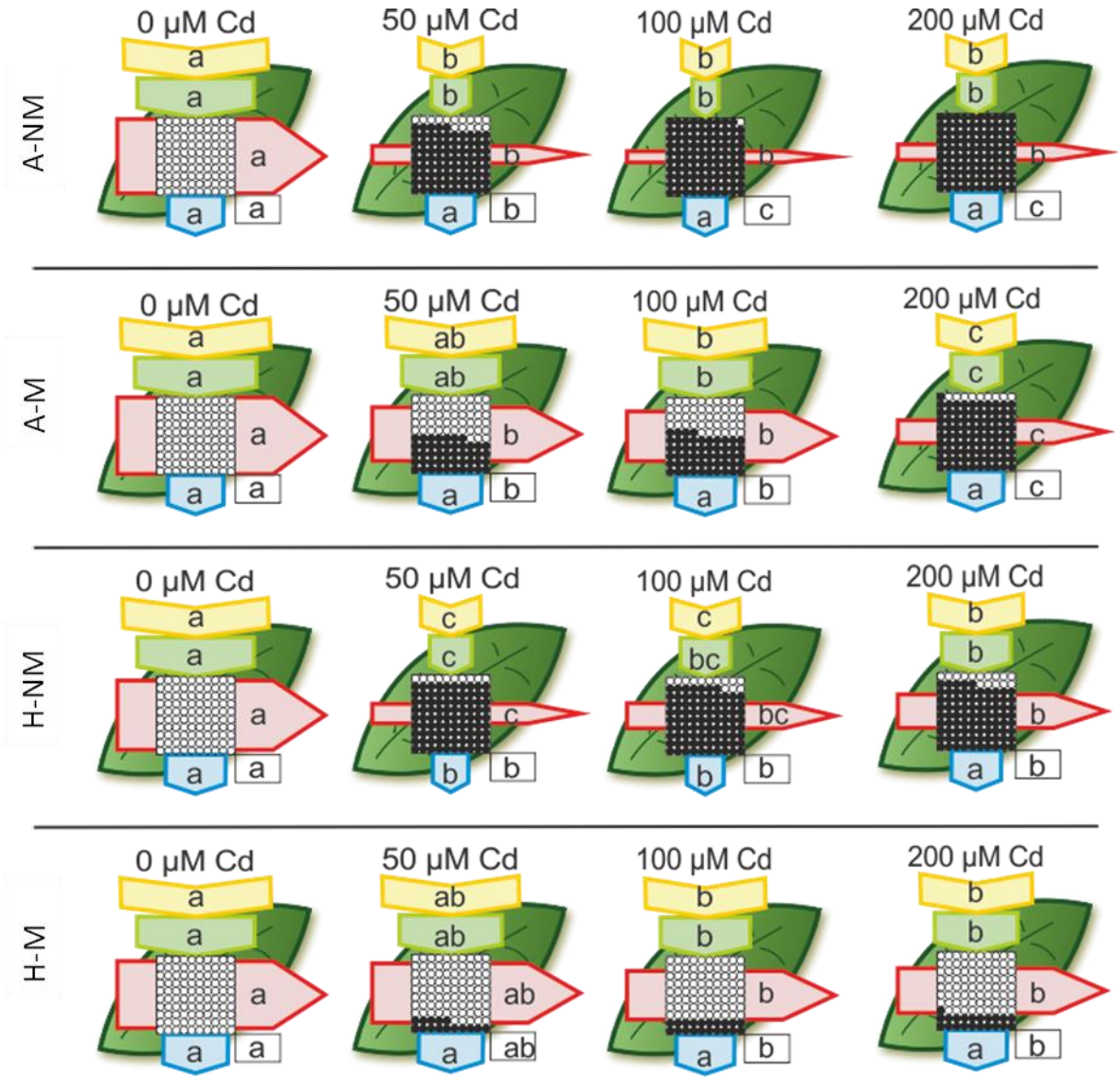

Figure S7. Leaf model showing the phenomenological energy fluxes per the excited cross sections (CS) of the leaves of the *A. arenosa* and *A. halleri* plants treated with different concentrations of Cd. Each relative value of the measured parameters is the mean (n = 20) and the width of each arrow corresponds to the intensity of the flux. Yellow arrow – ABS/CS, absorption flux per CS approximated; green arrow – TR/CS, trapped energy flux per CS; red arrow – ET/CS, electron transport flux per CS; blue arrow – DI/CS, dissipated energy flux per CS; circles inscribed in squares – RC/CS, % of active/inactive reaction centers. White circles inscribed in squares represent reduced Q_A reaction centers (active), black circles represent non-reducing Q_A reaction centers (inactive), 100% of the active reaction centers responded with the highest mean value observed in the control conditions. Means followed by the same letter for each parameter in a row are not significantly different from each other using the HSD test ($P < 0.05$). Letters are inscribed into arrows, except for RC/CS, where they are placed in a box in the bottom right corner of the square with circles.

Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

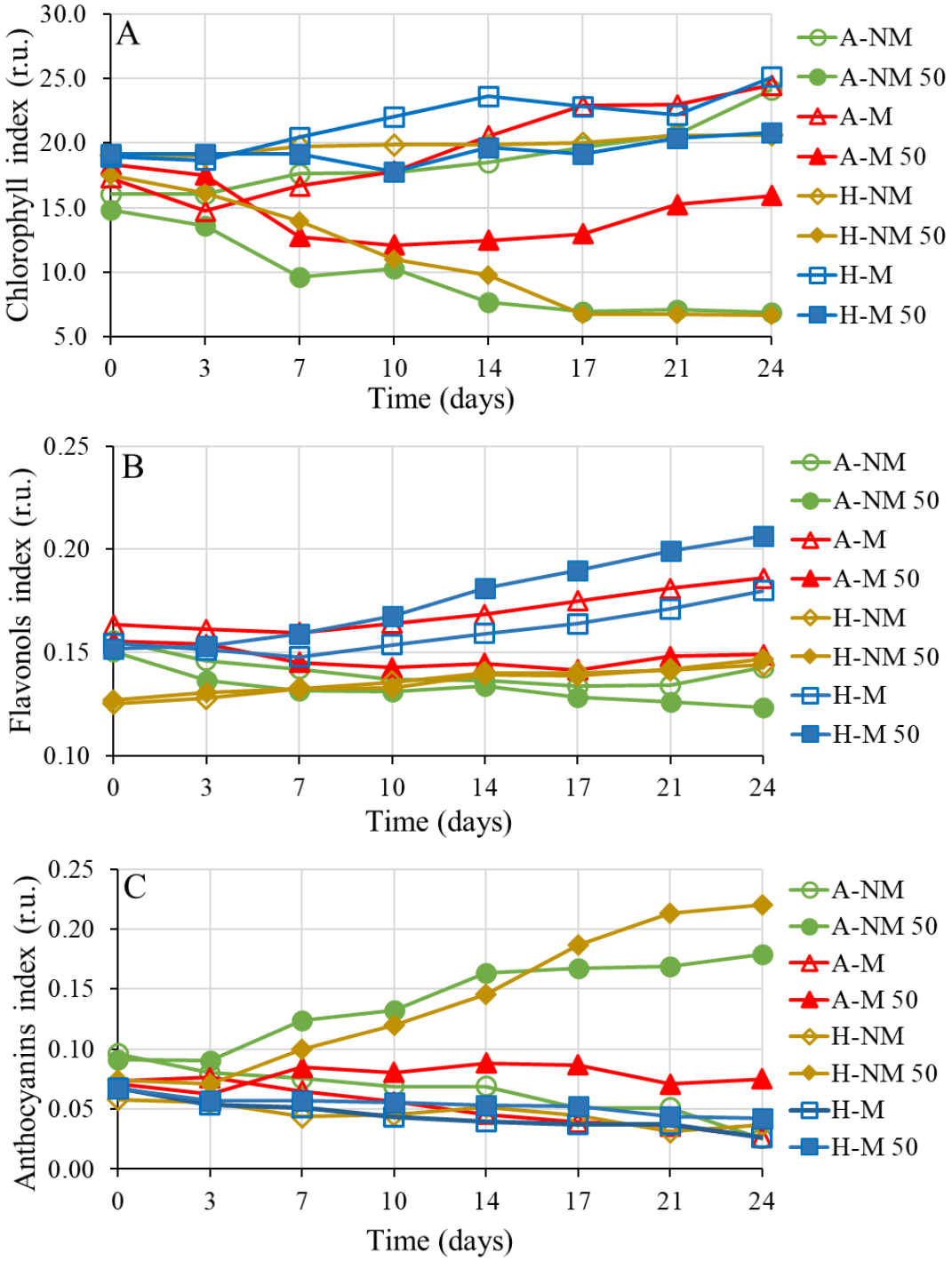

Figure S8. Changes over time in chlorophyll (A), flavonol (B) and anthocyanin (C) content indices in leaves of *A. arenosa* and *A. halleri* plants in the control medium and treated with Cd at the concentration of 50 µM (50 – treatment with 50 µM of Cd). Values are means (n= 20). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous). r.u., Relative units.

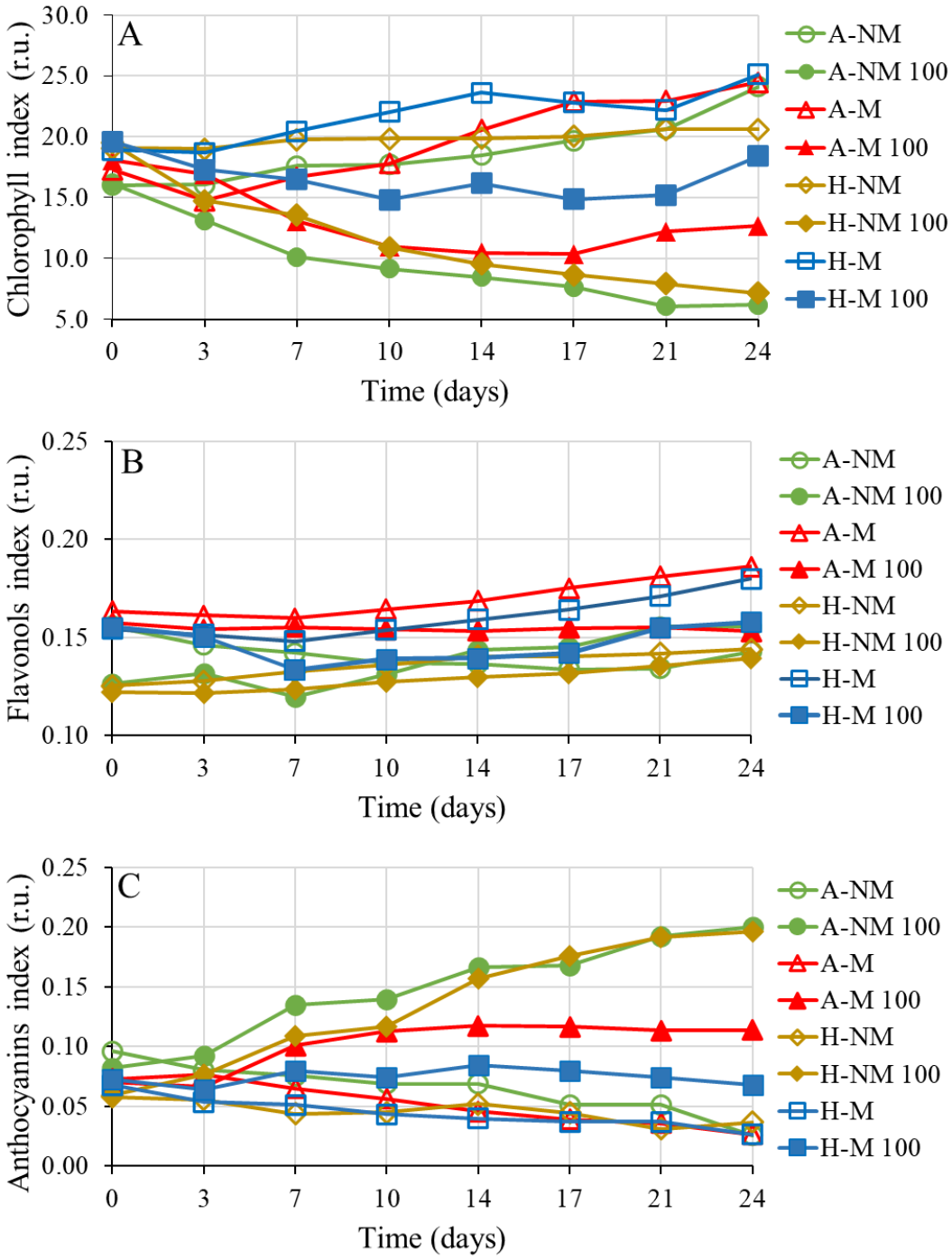

Figure S9. Changes over time in chlorophyll (A), flavonol (B) and anthocyanin (C) content indices in leaves of *A. arenosa* and *A. halleri* plants in the control medium and treated with Cd at the concentration of 100 µM (100 – treatment with 100 µM of Cd). Values are means (n= 20). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous). r.u., Relative units.

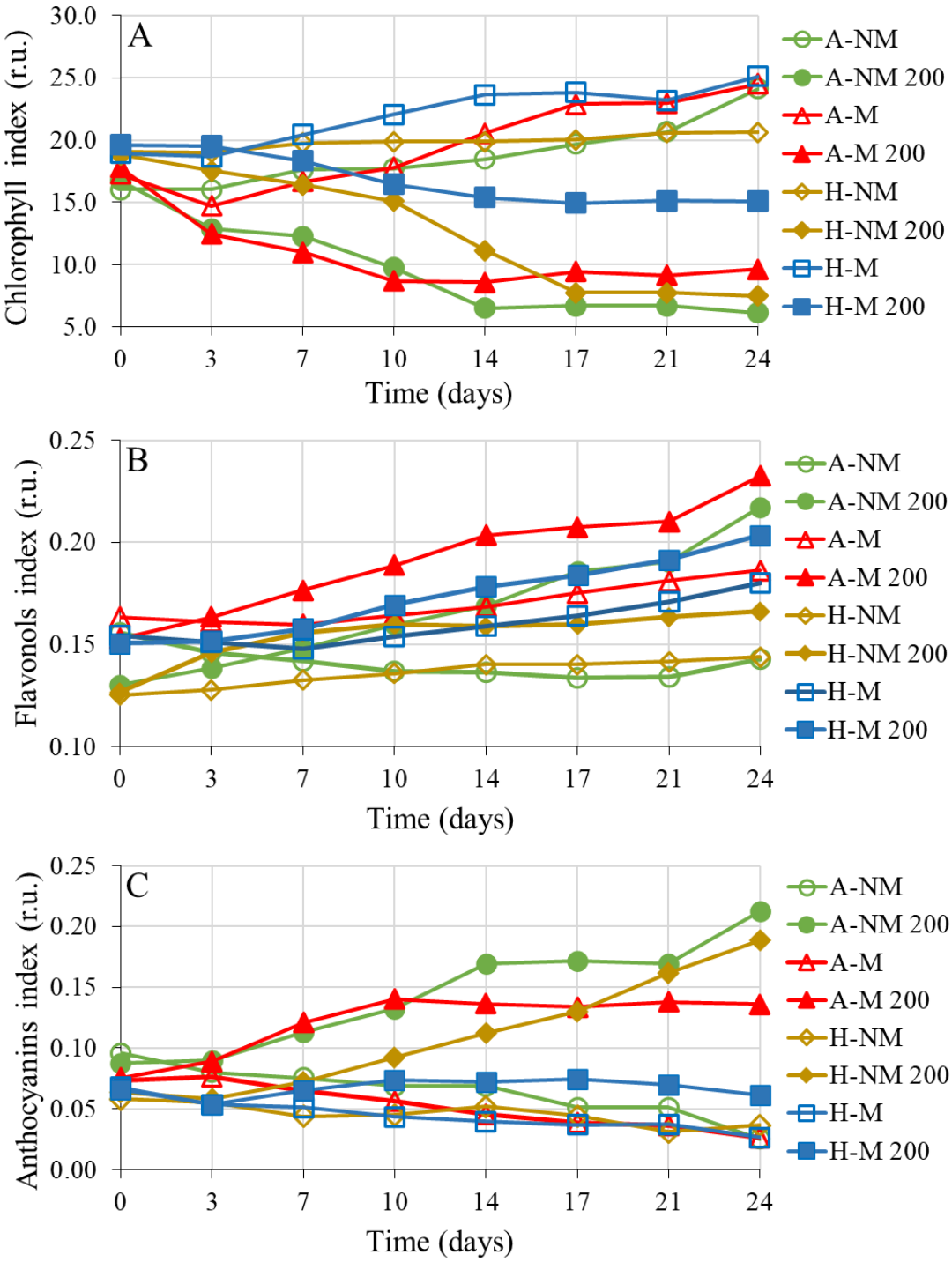

Figure S10. Changes over time in chlorophyll (A), flavonol (B) and anthocyanin (C) content indices in leaves of *A. arenosa* and *A. halleri* plants in the control medium and treated with Cd at the concentration of 200 µM (200 – treatment with 200 µM Cd). Values are means (n= 20). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous). r.u., Relative units.

Table S1. Geographical characteristics of the sites and ploidy level of populations

Species	*A. arenosa*		*A. halleri*	
Site localization	Klucze	Piekary Śląskie	Niepołomice	Piekary Śląskie
Edaphic type and names of the populations				
Name of population	A-NM	A-M	H-NM	H-M
Edaphic type	non-metalliferous	metalliferous	non-metalliferous	metalliferous
Latitude	50°20'03.5"N	50°22'00.6"N	50°06'35.6"N	50°22'00.6"N
Longitude	19°35'26.3"E	18°58'18.4"E	20°21'40.3"E	18°58'18.4"E
Habitat	pine forest edge	meager grassland	alluvial forest	meager grassland
DNA content and ploidy level of populations				
Approximate 2C DNA content	0.808 pg	0.804 pg	0.49 pg	0.48 pg
Ploidy level	tetraploid	tetraploid	diploid	diploid

M – metallicolous; NM – non-metallicolous; Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

Table S2. The shoot:soil bioavailable metal concentration ratio of *A. arenosa* and *A. halleri in situ*

Species	*A. arenosa*		*A. halleri*	
Name of population	A-NM	A-M	H-NM	H-M
Shoot:Soil metal concentration ratio				
Cd	22.2 ± 6.1 b	16.7 ± 1.0 b	963 ± 199 a	17.4 ± 1.7 b
Zn	45.5 ± 19.4 b	35.3 ± 1.5 b	539 ± 117 a	50.5 ± 5.9 b

M – metallicolous; NM – non-metallicolous; presented data are means ± SE (n = 8). Means followed by the same letter in a row are not significantly different from each other using the HSD test ($P \leq 0.05$). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

Table S3. Abbreviations and definitions of photosynthesis parameters

General terminology	
PSII	Photosystem II
PSI	Photosystem I
OJIP	Transient of chlorophyll *a* fluorescence rise induced during a dark-to-strong light transition, where O is equivalent to F_0 and P is equivalent to F_m
CS	Excited cross section of leaf
RC	Reaction center of PSII
Q_A	Primary quinone electron acceptor of PSII
FNR	Ferredoxin-$NADP^+$ Reductase
OEC	Oxygen Evolving Complex
Fluorescence parameters	
F_0	Minimal fluorescence, when all PSII RCs are open (at t = 0)
F_m	Maximal fluorescence, when all PSII RCs are closed
$F_v = F_m-F_0$	Maximal variable fluorescence
F_t	Fluorescence at time t
$V_t = (F_t-F_0)/(F_m-F_0)$	Relative variable fluorescence at time t
V_{tF}	Relative variable fluorescence at time t for reference population
$\Delta V_t = (F_t-F_0)/(F_m-F_0) - V_{tF}$	$V_t - V_{tF}$
T_{fm}	Time (in seconds) to reach maximal fluorescence (F_m)
Yields or flux ratios	
$\varphi D_0 = F_0/F_m$	Quantum yield (at t = 0) of energy dissipation
$\varphi P_0 = [1-(F_0/F_m)]$	Maximum quantum yield of primary photochemistry (at t = 0)
$\Psi E_0 = (1-V_J)$	Probability (at t = 0) that a trapped excition moves an electron into the electron transport chain beyond Q_A
$\varphi E_0 = [1-(F_0/F_m)](1-V_J)$	Quantum yield of electron transport (at t = 0)
$\delta R_0 = (1-V_I)/(1-V_J)$	Probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side
$\varphi R_0 = (1-F_0/F_m)(1-V_I)$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side
Phenomenological energy fluxes (per excited cross section of leaf)	
ABS/CS	Absorption flux pre CS
TR/CS	Trapped energy flux per CS
ET/CS	Electron transport per CS
DI/CS	Dissipation energy flux per CS
RC/CS	% of active reaction centers per CS in comparison with initial state

Table S4. Characteristic of the photosynthetic apparatus

Species	*A. arenosa*		*A. halleri*	
Name of population	A-NM	A-M	H-NM	H-M
PSII characteristics (relative units)				
T_{Fm}	193.3 ± 11.9 c	317.5 ± 22.0 b	398.8 ± 21.3 a	234.2 ± 6.0 c
Area	321,500 ± 19,500 b	241,700 ± 7,500 c	373,700 ± 9,500 a	364,300 ± 10,600 al
F_0	5,490 ±170 a	4,880 ± 90 b	4,997 ± 41 b	5,292 ± 85 ab
F_m	25,840 ± 520 b	24,500± 690 b	30,550 ± 250 a	24,170 ± 450 b
F_v	20,350 ± 614 b	19,620 ± 600 b	25,550 ± 217 a	18,880 ± 449 b
φP_0	0.78 ± 0.01 b	0.80 ± 0.00 b	0.84 ± 0.00 a	0.78 ± 0.01 b
ψE_0	0.54 ± 0.02 a	0.51 ± 0.00 ab	0.48 ± 0.00 b	0.39 ± 0.01 c
φE_0	0.43 ± 0.02 a	0.40 ± 0.01 a	0.40 ± 0.00 a	0.30 ± 0.01 b
δR_0	0.43 ± 0.03 a	0.42 ± 0.01 a	0.18 ± 0.00 b	0.46 ± 0.01 a
φR_0	0.17 ± 0.01 a	0.15 ± 0.00 b	0.07 ± 0.00 c	0.14 ± 0.00 b

M – metallicolous; NM – non-metallicolous; presented data are means ± SE (n = 24). Means followed by the same letter in a row are not significantly different from each other using the HSD test ($P \leq 0.05$). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

Parameters: T_{Fm} – time (in ms) to reach maximal fluorescence F_m; Area – the area above the chlorophyll fluorescence curve between F0 and Fm (reflecting the size of the plastoquinone pool); F_0 – minimal fluorescence, when all of the PSII RCs are open (at t = 0); F_m – maximal fluorescence, when all of the PSII RCs are closed; F_v – maximum variable fluorescence; φP_0 – maximum quantum yield of the primary PSII photochemistry; ΨE_0 – probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A–; φE_0 – quantum yield for electron transport from Q_A− to plastoquinone; δR_0 – probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side; φR_0 – quantum yield for the reduction of the end electron acceptors at the PSI acceptor side; φD_0 – quantum yield (at t = 0) of energy dissipation.

Table S5. Accumulation of elements in roots of *A. arenosa* and *A. halleri* under different Cd treatment (µg g^{-1} DW).

Species		*A. arenosa*		*A. halleri*	
Name of population		A-NM	A-M	H-NM	H-M
Accumulation of elements in roots					
Ca	0 µM Cd	7,700 ± 711 aA	9,830 ± 467 aA	7,340 ± 859 aA	8,490 ± 647 aB
	50 µM Cd	9,900 ± 791 aA	11,100 ± 924 aA	9,110 ± 708 aA	8,140 ± 387 aB
	100 µM Cd	7,550 ± 1110 bA	7,920 ± 669 bA	17,610 ± 4170 aA	6,690 ± 419 bB
	200 µM Cd	7,630 ± 1090 aA	11,430 ± 1980 aA	13,120 ± 3690 aA	10,250 ± 423 aA
Cd	0 µM Cd	15.2 ± 1.29 aC	14.3 ± 1.01 aD	5.60 ± 0.51 bC	7.52 ± 0.85 bD
	50 µM Cd	6,990 ± 398 aB	3,970 ± 298 bC	3,810 ± 306 bcB	2,560 ± 154 cC
	100 µM Cd	18,480 ± 4280 aA	10,750 ± 1170 abB	8,890 ± 1790 abAB	7,120 ± 544 bB
	200 µM Cd	13,100 ± 1020 bA	20,620 ± 1530 aA	10,210 ± 1220 bA	12,150 ± 388 bA
Cu	0 µM Cd	86.3 ± 16.9 aB	100 ± 14.9 aB	94.3 ± 19.2 aB	48.9 ± 3.81 aC
	50 µM Cd	129 ± 7.43 bB	204 ± 17.8 aAB	62.1 ± 11.2 cB	107 ± 8.55 bcBC
	100 µM Cd	236 ± 25.3 aA	204 ± 26.4 aAB	137 ± 28.5 aB	146 ± 23.9 aB
	200 µM Cd	273 ± 22.1 aA	367 ± 83.7 aA	331 ± 69.0 aA	228 ± 20.7 aA
Fe	0 µM Cd	10,060 ± 576 aA	10,020 ± 684 aB	9,910 ± 1,030 aB	10,880 ± 474 aA
	50 µM Cd	17,350 ± 1,540 aA	9,160 ± 506 cB	14,360 ± 613 abB	11,410 ± 672 bcA
	100 µM Cd	25,320 ± 7,620 abA	8,380 ± 991 bB	28,890 ± 4,790 aA	12,950 ± 643 abA
	200 µM Cd	17,570 ± 2,750 aA	14,780 ± 1,800 aA	16,690 ± 620 aB	12,840 ± 406 aA
Mg	0 µM Cd	2,260 ± 158 aA	1,440 ± 70.4 bB	1,420 ± 127 bC	1,400 ± 113 bB
	50 µM Cd	2,600 ± 143 aA	2,070 ± 232 abB	2,060 ± 54.6 abBC	1,700 ± 67.0 bB
	100 µM Cd	2,830 ± 594 bA	1,860 ± 92.0 bB	5,640 ± 548 aA	1,890 ± 98.5 bB
	200 µM Cd	4,270 ± 965 aA	3,120 ± 254 aA	3,540 ± 714 aB	3,130 ± 443 aA
Mn	0 µM Cd	110 ± 15.3 bA	445 ± 63.4 aA	284 ± 62.7 abA	158 ± 9.43 bA
	50 µM Cd	69.6 ± 5.78 aA	49.6 ± 2.97 abB	54.1 ± 6.98 abB	38.1 ± 3.52 bB
	100 µM Cd	98.5 ± 27.1 abA	42.0 ± 1.89 bB	140 ± 19.8 aAB	43.0 ± 8.17 bB
	200 µM Cd	88.0 ± 20.9 aA	58.3 ± 2.20 aB	105 ± 24.5 aB	51.8 ± 7.13 aB
Zn	0 µM Cd	707 ±114 aA	256 ± 36.6 bA	194 ± 54.1 bA	65.5 ± 10.9 bA
	50 µM Cd	541 ± 125 aA	258 ± 27.7 bA	142 ± 19.7 bA	77.2 ± 15.9 bA
	100 µM Cd	1,160 ± 408 aA	285 ± 27.4 bA	193 ± 45.3 bA	32.6 ± 9.24 cA
	200 µM Cd	242 ± 40.7 abA	389 ± 67.7 aA	125 ± 18.3 bcA	54.9 ± 10.7 cA

M – metallicolous; NM – non-metallicolous; presented data are means ± SE (n = 6). Means followed by the same lower case letter in a row and capital letter in a column (for single element) are not significantly different from each other using the HSD test ($P \leq 0.05$). Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous).

Table S6. Characteristic of the photosynthetic apparatus

Name of population and concentration of Cd in medium				
		A-NM		
Concentration of Cd (μM)	0	50	100	200
PSII characteristics (relative units)				
t_{Fm}	400 ± 31 a	272 ± 28 b	334 ± 31 ab	276 ± 25 b
Area	618,900 ± 42,300 a	135,000 ± 26,600 b	117,200 ± 14,300 b	165,100 ± 19,300 b
F_0	6,133 ± 161 a	9,027 ± 1,330 a	7,894 ± 744 a	8,184 ± 999 a
F_m	31,960 ± 690 a	20,280 ± 2,480 b	16,450 ± 1,240 b	20,950 ± 1,920 b
F_v	25,830 ± 570 a	11,260 ± 1,780 b	8,560 ± 860 b	12,760 ± 1,530 b
φP_0	0.81 ± 0.00 a	0.52 ± 0.04 b	0.52 ± 0.03 b	0.60 ± 0.04 b
ψE_0	0.63 ± 0.01 a	0.29 ± 0.05 c	0.36 ± 0.02 bc	0.44 ± 0.02 b
φE_0	0.51 ± 0.01 a	0.18 ± 0.03 c	0.20 ± 0.02 bc	0.27 ± 0.02 b
δR_0	0.28 ± 0.02 a	0.24 ± 0.02 a	0.24 ± 0.02 a	0.20 ± 0.02 a
φR_0	0.15 ± 0.01 a	0.04 ± 0.01 b	0.04 ± 0.00 b	0.05 ± 0.01 b
		A-M		
t_{Fm}	463 ± 30 a	316 ± 28 a	334 ± 38 a	386 ± 53 a
Area	623,100 ± 22,100 a	355,600 ± 33,500 b	365,100 ± 35,800 b	219,100 ± 36,900 c
F_0	6,180 ± 115 b	8,481 ± 792 a	7,894 ± 506 ab	8,677 ± 576 a
F_m	32,920 ± 410 a	31,460 ± 1.230 a	29,300 ± 1,240 a	22,520 ± 1,350 b
F_v	26,740 ± 330 a	22,980 ± 1,540 ab	21,580 ± 1,450 b	13,840 ± 1,400 c
φP_0	0.81 ± 0.00 a	0.71 ± 0.04 a	0.72 ± 0.03 a	0.58 ± 0.03 b
ψE_0	0.63 ± 0.01 a	0.50 ± 0.03 bc	0.51 ± 0.03 b	0.40 ± 0.02 c
φE_0	0.51 ± 0.01 a	0.38 ± 0.03 b	0.38 ± 0.03 b	0.25 ± 0.03 c
δR_0	0.27 ± 0.01 a	0.20 ± 0.01 b	0.21 ± 0.01 b	0.22 ± 0.02 b
φR_0	0.14 ± 0.00 a	0.08 ± 0.01 b	0.08 ± 0.01 b	0.05 ± 0.01 b
		H-NM		
t_{Fm}	533 ± 27 a	253 ± 7 b	356 ± 42 b	344 ± 31 b
Area	670,400 ± 27,300 a	189,000 ± 18,000 b	218,200 ± 35,700 b	209,100 ± 15,100 b
F_0	6,710 ± 180 a	4,824 ± 478 b	4,671 ± 485 b	5,324 ± 231 ab
F_m	32,820 ± 650 a	15,540 ± 1,270 b	16,890 ± 1,990 b	18,330 ± 870 b
F_v	26,110 ± 480 a	10,710 ± 950 b	12,210 ± 1,570 b	13,010 ± 1,500 b
φP_0	0.80 ± 0.00 a	0.69 ± 0.02 b	0.70 ± 0.02 b	0.70 ± 0.02 b
ψE_0	0.60 ± 0.01 a	0.45 ± 0.01 b	0.48 ± 0.02 b	0.43 ± 0.03 b
φE_0	0.48 ± 0.01 a	0.32 ± 0.02 b	0.34 ± 0.02 b	0.31 ± 0.03 b
δR_0	0.31 ± 0.02 a	0.32 ± 0.01 a	0.26 ± 0.02 a	0.27 ± 0.02 a
φR_0	0.15 ± 0.01 a	0.10 ± 0.01 b	0.09 ± 0.01 b	0.08 ± 0.00 b
		H-M		
t_{Fm}	560 ± 19 a	383 ± 37 b	478 ± 47 ab	418 ± 51 ab
Area	551,000 ± 24,500 a	724,700 ± 26,700 a	409,800 ± 48,300 a	392,900 ± 31,500 a
F_0	7,463 ± 213 a	8,790 ± 818 a	7,726 ± 547 a	7,078 ± 489 a
F_m	34,380 ± 470 a	33,250 ± 1,300 ab	29,130 ± 920 bc	26,700 ± 1,420 c
F_v	26,910 ± 425 a	24,460 ± 1,690 ab	21,400 ± 1,370 b	19,610 ± 1,500 b
φP_0	0.78 ± 0.01 a	0.72 ± 0.04 a	0.72 ± 0.03 a	0.72 ± 0.03 a
ψE_0	0.57 ± 0.01 a	0.52 ± 0.04 a	0.50 ± 0.03 a	0.51 ± 0.03 a
φE_0	0.45 ± 0.01 a	0.39 ± 0.03 a	0.37 ± 0.03 a	0.38 ± 0.03 a
δR_0	0.24 ± 0.01 a	0.24 ± 0.02 a	0.23 ± 0.01 a	0.28 ± 0.03 a
φR_0	0.11 ± 0.01 a	0.10 ± 0.01 a	0.09 ± 0.01 a	0.10 ± 0.01 a

Presented data are means ± SE (n = 20). Means followed by the same letter in a row are not significantly different from each other using the HSD test ($P \leq 0.05$). M – metallicolous; NM – non-metallicolous. Abbreviations of the plant population names: A-NM – *A. arenosa* from Klucze (non-metalliferous); A-M – *A. arenosa* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); H-M – *A. halleri* from Piekary Śląskie (metalliferous). Parameters: T_{Fm} – time (in ms) to reach maximal fluorescence F_m; Area – the area above the chlorophyll fluorescence curve between F_0 and F_m (reflecting the size of the plastoquinone pool); F_0 – minimal fluorescence, when all of the PSII RCs are open (at t = 0); F_m – maximal fluorescence, when all of the PSII RCs are closed; F_v – maximum variable fluorescence; φP_0 – maximum quantum yield of the primary PSII photochemistry; ΨE_0 – probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A–; φE_0 – quantum yield for electron transport from Q_A− to plastoquinone; δR_0 – probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side; φR_0 – quantum yield for the reduction of the end electron acceptors at the PSI acceptor side; φD_0 – quantum yield (at t = 0) of energy dissipation.

13.2. Supplemental materials for Chapter II

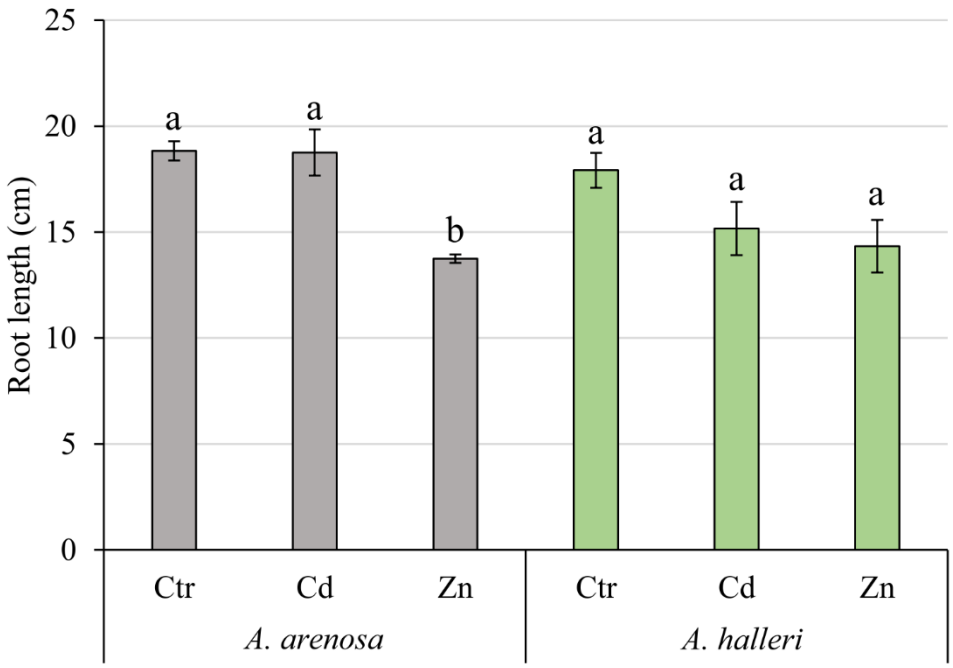

Figure S1. Length of roots of *A. arenosa* (grey) and *A. halleri* (green) at the end of experiment (120 h). Ctr – control; Cd – 1.0 mM Cd treatment; Zn – 5.0 mM Zn treatment. Values are means ± SE (n = 6). Means followed by the same letter for each species are not significantly different from each other using the HSD test ($P < 0.05$).

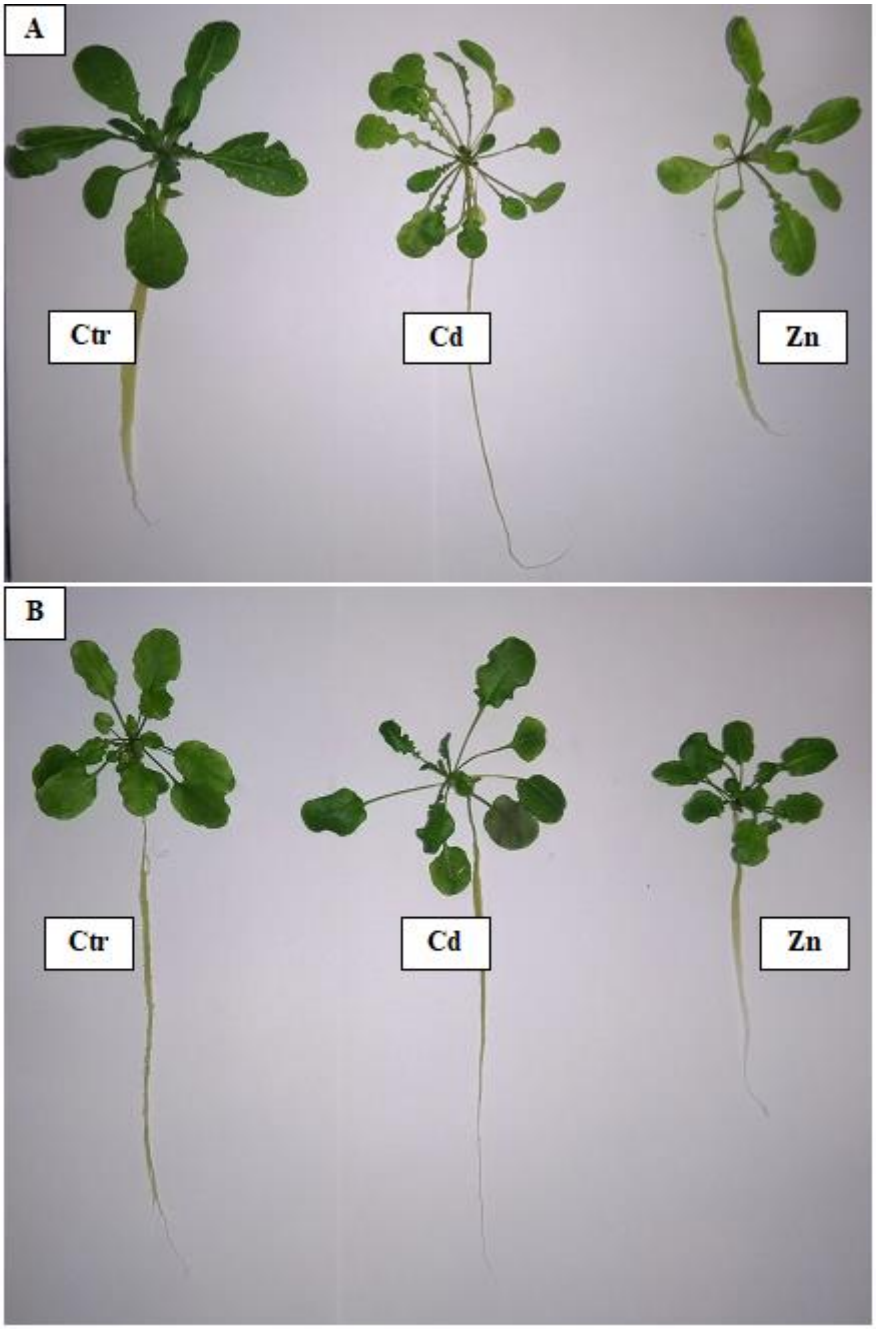

Figure S2. The pictures of *A. arenosa* (A) and *A. halleri* (B) at the end of experiment; Ctr – control; Cd – 1.0 mM Cd treatment; Zn – 5.0 mM Zn treatment.

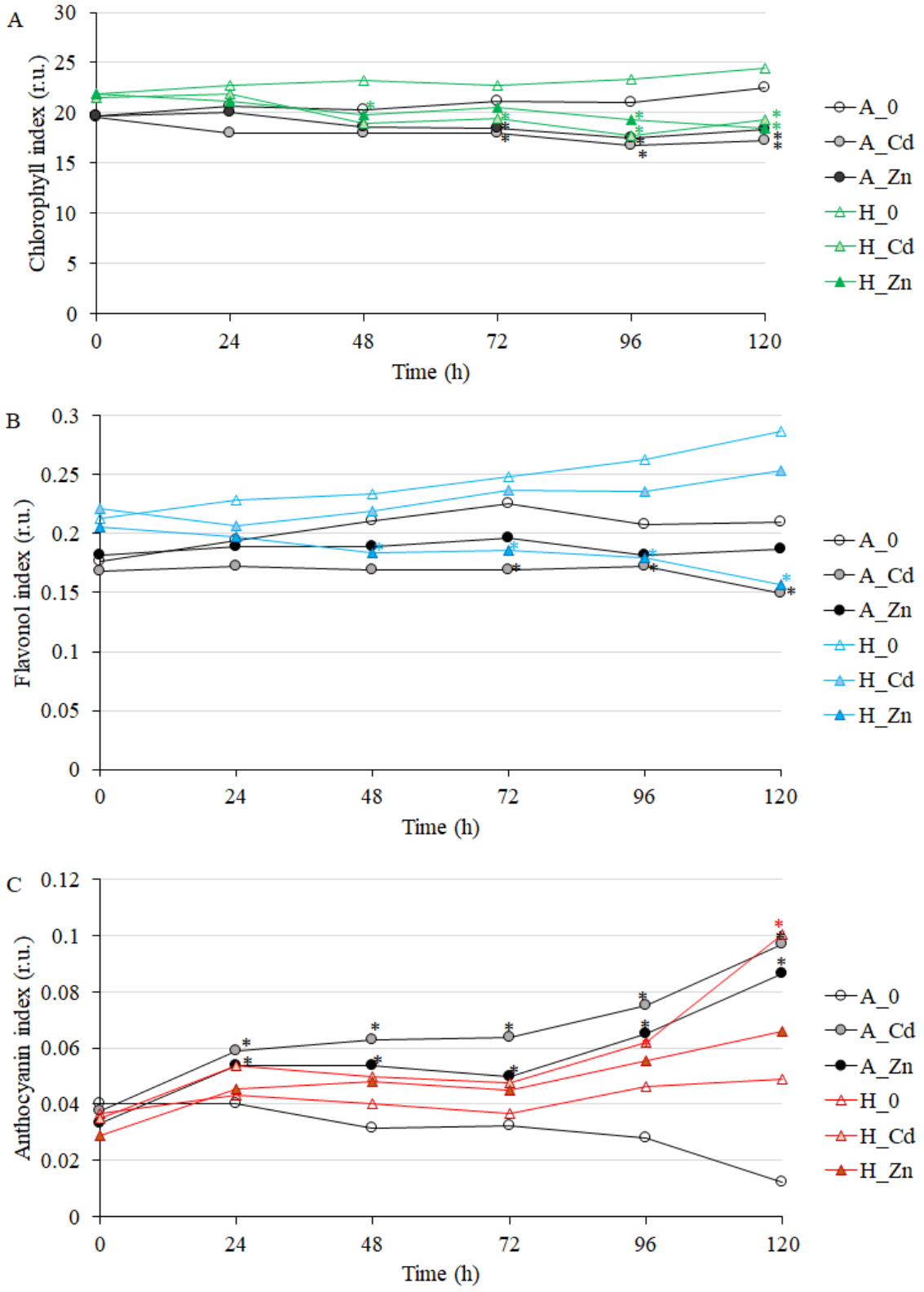

Figure S3. Changes in chlorophyll (A), flavonol (B) and anthocyanin (C) index (relative units) in *A. arenosa* (A_treatment) and *A. halleri* (H_treatment) leaves in control and under Cd or Zn treatment during 120 hours. Values are means (n = 30). Asterisk (*) means significant difference compared to control using Tukey HSD test ($P < 0.05$).

Table S1 Summary of two-way ANOVA analysis for effect of treatment, plant species and interaction between these two factors on different physiological parameters

Factors	Shoot biomass	Root biomass	Root length	H_2O_2 concentration	MDA content	Catalase activity	Chlorophyll index	Flavonol index	Anthocyanin index	Intracellular CO_2 concentration	Photosynthetic rate	Stomatal conductance	Transpiration rate
Species	X	X	–	–	X	X	X	X	–	X	X	X	X
Treatment	X	X	X	X	X	X	X	X	X	X	X	X	X
Species/ treatment	–	X	–	–	–	X	–	X	X	–	X	X	X

"Species" (*A. arenosa*, *A. halleri*); "Treatment" (Control, 1.0 mM Cd, 5.0 mM Zn); "Species/treatment" – interaction between these two factors. "X" means that the factors have statistically significant effect on a measured parameter (p <0.05).

Table S2. Abbreviations and definitions of photosynthesis parameters

General terminology	
PSII	Photosystem II
PSI	Photosystem I
OJIP	Transient of chlorophyll *a* fluorescence rise induced during a dark-to-strong light transition, where O is equivalent to F_0 and P is equivalent to F_m
CS	Excited cross section of leaf
RC	Reaction center of PSII
Q_A	Primary quinone electron acceptor of PSII
FNR	Ferredoxin-$NADP^+$ Reductase
OEC	Oxygen Evolving Complex
MDA	Malondialdehyde
CAT	Catalase
Fluorescence parameters	
F_0	Minimal fluorescence, when all PSII RCs are open (at t = 0)
F_m	Maximal fluorescence, when all PSII RCs are closed
$F_v = F_m - F_0$	Maximal variable fluorescence
F_t	Fluorescence at time t
$V_t = (F_t - F_0)/(F_m - F_0)$	Relative variable fluorescence at time t
V_{tE}	Relative variable fluorescence at time t for control
$\Delta V_t = (F_t - F_0)/(F_m - F_0) - V_{tE}$	$= V_t - V_{tE}$
Yields or flux ratios	
$\varphi D_0 = F_0/F_m$	Quantum yield (at t = 0) of energy dissipation
$\varphi P_0 = [1-(F_0/F_m)]$	Maximum quantum yield of primary photochemistry (at t = 0)
$\Psi E_0 = (1-V_J)$	Probability (at t = 0) that a trapped excition moves an electron into the electron transport chain beyond Q_A
$\varphi E_0 = [1-(F_0/F_m)](1-V_J)$	Quantum yield of electron transport (at t = 0)
$\delta R_0 = (1-V_I)/(1-V_J)$	Probability with which an electron from the intersystem electron carriers will move to reduce the end acceptors at the PSI acceptor side
$\varphi R_0 = (1-F_0/F_m)(1-V_I)$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side
Phenomenological energy fluxes (per excited cross section of leaf)	
ABS/CS	Absorption flux pre CS
TR/CS	Trapped energy flux per CS
ET/CS	Electron transport per CS
DI/CS	Dissipation energy flux per CS
RC/CS	% of active reaction centers per CS in comparison with initial state
Gas exchange parameters	
A	Photosynthesis rate
C_i	Intracellular CO_2 concentration
g_s	Stomatal conductance
E	Transpiration rate

13.3. Supplemental materials for Chapter III

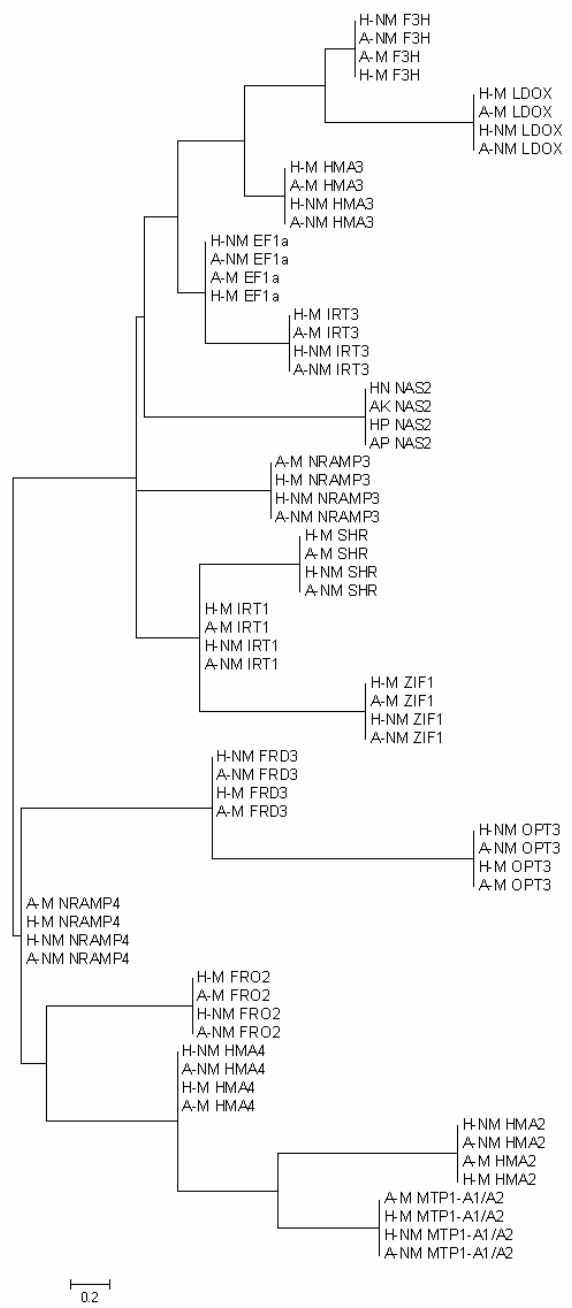

Figure S1. Phylogenetic tree of DNA sequences amplified in qRT-PCR. Abbreviations of the plant populations names: H-M - *A. halleri* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); A-M - *A. arenosa* from Piekary Śląskie (metalliferous); A-NM – *A. arenosa* from Klucze (metalliferous).

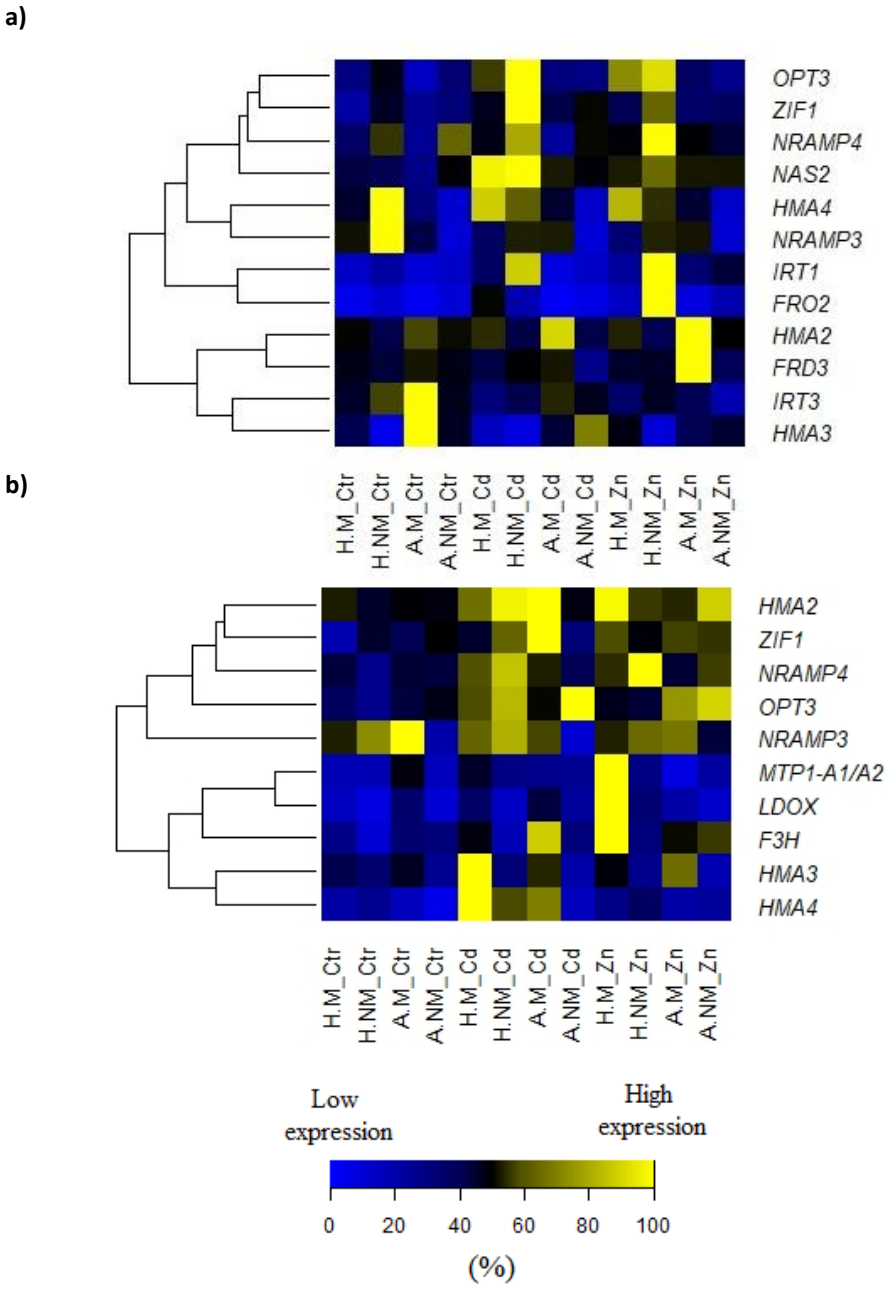

Figure S2. Expression of genes in roots (a) and shoots (b), involved in metal uptake, translocation and detoxification in metallicolous (H-M) and non-metallicolous (H-NM) *Arabidopsis halleri* and metallicolous (A-M) and non-metallicolous (A-NM) *Arabidopsis arenosa* population after 10 d in control (Ctr) and under 5 µM Cd (Cd) and 150 µM Zn treatment (Zn). The expression values were calculated as a percentage related to the sample showing the highest expression value for each gene (100% and 0% for yellow and blue colors respectively) within HP, HN, AP and AK (control (Ctr), cadmium (Cd) and zinc (Zn) treatments). Gene names are also reported.

Table S1. Geographical characteristics of the sites

Species	*A. halleri*		*A. arenosa*	
Site localisation	Piekary Śląskie	Niepołomice	Piekary Śląskie	Klucze
Populations names and edaphic type				
Name of population	H-M	H-NM	A-M	A-NM
Edaphic type	metalliferous	non-metalliferous	metalliferous	non-metalliferous
Latitude	50°22'00.6"N	50°06'35.6"N	50°22'00.6"N	50°20'03.5"N
Longitude	18°58'18.4"E	20°21'40.3"E	18°58'18.4"E	19°35'26.3"E
Habitat	meagre grassland	alluvial forest	meagre grassland	pine forest edge

Abbreviations of the plants populations names: H-M - *A. halleri* from Piekary Śląskie (metalliferous); H-NM – *A. halleri* from Niepołomice (non-metalliferous); A-M - *A. arenosa* from Piekary Śląskie (metalliferous); A-NM – *A. arenosa* from Klucze (metalliferous).

Table S2. Sequences of primers

Primers used for RT-qPCR analysis		
Target Gene	Forward	Reverse
HMA4	CTCACTGCCTCTCTCAACCTTT	GGGAAGAAGCCGAGAAGAAAGA
HMA3	TTGTCGATATGGGGTTGCTCAA	CGTCTCGGTTATCTCCTGTGAG
HMA2	GTTCGGACCATAGTCACTCTGG	TACCACAACACCCTGAGTCATG
MTP1-A1/A2	AGTGGTGAACATCATAATGGCTGTTC	TCGATTTGTCCAACAGTTGCTCAG
IRT1	TCCTCCAGGCTGAGTATACGAA	CGATCCCTAACGCTATTCCGAA
IRT3	ACATGGCTTTGGTGGATCTCAT	AATGGCAAGGGAAGACATGAGT
OPT3	TGCAAAGCTACAAGAAAGTGCC	CTGACTCCTTCCACACGAAAGA
FRO2	CCAACACTAACCTACCGAACCA	ATCCCAAACAAGCTACGACCAT
FRD3	TCGGTGATTTGCAGTTCGGA	GCTGCTGCTAAGGTCTGACA
ZIF1	AAGTACGGAGGTCTGAGCTACT	CCAGAACAGGTCCTAGCAGTTT
NRAMP3	AACAGCGATGGGTCTATTGGTT	CCAATCAAAGCCAATTCAGCCA
NRAMP4	TTGGAGCATTGGTCCCTAAG	ATTGCACAAGTGCTGAATGC
LDOX	GTGAAGAAAGCCGGGGAAGA	TCCGCAAGCGTTGTTAGCTA
F3H	GGGAGAGGCTGTGCAAGATT	CTCCTCCGTCACTTTCACCC
EF1a	TGAGCACGCTCTTCTTGCTTTCA	GGTAGTGGCATCCATCTTGTTACA
SHR	GTCGGAGAAGAAGAAGGTGGCT	CCATGACTCGAAGCAAACCCTAAA
Primers used for amplicon sequencing		
Target Gene	Forward	Reverse
HMA4	ACCTCAATCTTCTTCTCAAAGTTCT	CGCCATTTTCGGAGAAGAGACGC
HMA3	CGGAAGTTTCAACCTTATTGAC	AGGGCTGCGTCTCGGTTA
HMA2	AAGACTCAAGTGAAAGTGATGCAA	TGTTGATGTGGTTGTTGACTCT
MTPA1/A2	GAGGACCAGCACCATGCTCATGG	CTGTGATAGCCCAAATGTGAAGC
IRT1	CCATTAAAGGACTCATCGCAGCT	TGAGAAGTCCAACCGTGATC
IRT3	TCTCAGCCGGAATTCTTGTTT	GGAATCTTAGAGTCTAATTAAGCCCA

OPT3	ACACCACGAAGCTGGACATC	GACAATAAAGGCCAATGCAAAAGCAA
FRO2	AGTGACTAAGTTCTTGATGATGGTGA	TATGGTCTGACGACTTTCGATTC
FRD3	ATTGTTCGTCTGCCTCGCAAA	GAAAAGCAGCCATTGGTGTTG
ZIF1	TGCACTATGGGCTAACAGTC	GCTTAGACTGAGACCTGATAAGC
NRAMP3	TACTCGTTGTTATGGCTTCTTATGT	GCAATAGCACTACCAATAACTTCT
NRAMP4	ACCCAGTGGAACCGAACTC	TCTTTTTAGGATCGACTTCTCTCG
LDOX	TTGGATTGGGGAGTGATGCA	TCTCTCTTGTCTTCAGGATACACAAG
F3H	TGACTTCTTCGCTTTACCTCCGG	AGCTTCAGACAAAACCTCAAGAA
EF1a	TCTCTAAGGATGGTCAGACCC	TACCTAGCCTTGGAGTATTTGGG
SHR	TGATATCGAGTTTCAGAAGGTTAAGG	CTCTAGCATCAACCTCTCGTT