

Article

Heavy Metal Tolerance and Accumulation Potential of a Rare Coastal Species, *Anthyllis vulneraria* subsp. *maritima*

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Abstract: The aim of the present study was to explore heavy metal tolerance and accumulation potential in *Anthyllis vulneraria* subsp. *maritima* plants from coastal sand dunes in controlled conditions. Plants were established from seeds collected in coastal sand dunes and cultivated in substrates in greenhouse conditions. A gradual treatment with CdCl₂, PbOAc, CuSO₄, MnSO₄, and ZnSO₄ was performed until three final concentrations for each metal were reached. The number of leaves, their biomass, and biomass of roots were negatively affected by increasing concentrations of lead (Pb) and manganese (Mn) in substrate, but no negative effect was evident for cadmium (Cd), copper (Cu), and zinc (Zn). Visible effects of metal toxicity were evident for Pb-treated plants (appearance of thinner leaves, yellowing of older leaves), as well as for Mn-treated plants (reduced leaf size, curled leaves, red leaf venation). There was a significant decrease in water content in old leaves at high Pb and increasing Mn concentration, indicating accelerated leaf senescence. Increase in polyphenol oxidase activity in leaves was evident in all the plants treated with heavy metals. In contrast, an increase in peroxidase activity was evident only for plants treated with 50 and 100 mg L^{−1} Cd, 500 mg L^{−1} Pb, 200–1000 mg L^{−1} Mn, and 500 mg L^{−1} Zn. Metal accumulation potential for Cd and Cu was the highest in the roots, but for Pb, Mn, and Zn, more metal accumulated in old leaves. It can be concluded that *A. vulneraria* subsp. *maritima* plants are tolerant to high Cd, Cu, and Zn, but moderately susceptible to Pb and Mn. However, oxidative enzyme activity cannot be unequivocally used as a specific indicator of metal tolerance. In respect to phytoremediation potential, the plants have very good accumulation capacity for Pb, Mn, and Zn.

Keywords: *Anthyllis vulneraria*; heavy metals; metal accumulation; oxidative enzymes; tolerance



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1. Introduction

Plant tolerance to heavy metals has long been the subject of intense scientific interest [1]. Studies of metal tolerant wild plants have provided an opportunity to understand the mechanisms of tolerance and have led to the use of this knowledge in the establishment of metal-resistant crops [2,3]. On the other hand, wild plant species capable of growing in metal-contaminated soil could be used in the reclamation of degraded sites. More recently, plants that are not only tolerant to heavy metals, but also able to efficiently accumulate

these metals in the above-ground parts have attracted interest [4]. Such plants can be used in various phytoremediation systems for the removal of metal pollutants from soil and water.

Plants naturally occurring in metal-rich soils are known as “metallophytes” [5]. Metallophytes can be either metal excluders or accumulators, emphasizing genetical independence of the two phenomena, metal tolerance and accumulation [6,7]. The phenomenon of “hyperaccumulation” has received a lot of attention in recent decades [8]. Unique properties of native metal-hyperaccumulating plants allow them to accumulate heavy metals in above-ground plants at concentrations two to three orders of magnitude exceeding these of other plants growing in the same metal-contaminated soils [9]. At a physiological level, constitutive overexpression of genes coding transmembrane proteins is one of the most characteristic features of metal-hyperaccumulating plants [7,8].

While initially mostly species from metalliferous soils have been tested in order to find tolerant metal-accumulating species, later it was found that many halophyte species also have properties useful for phytoremediation [4]. On the other hand, physiological characteristics suitable for phytoremediation have also been found in many wetland species [3]. So far, only a few legume species (Fabaceae) have been characterized as metallophytes, including *Anthyllis vulneraria* [10–12], *Astragalus* spp. [13,14], *Coronilla juncea* [15], and *Lotus corniculatus* [11,12]. No hyperaccumulators have been found among the species of Fabaceae, though [16].

One of the legume species, *Anthyllis vulneraria* L., has attracted special attention in the context of the tolerance and accumulation of heavy metals. Taxonomy of genus *Anthyllis* has been the subject of long-standing scientific controversy [17]. *A. vulneraria*, widely distributed in Europe, represents a highly polymorphic species. While local botanists often consider *A. vulneraria* plants from coastal dunes of the Eastern Baltics a separate species, *Anthyllis maritima* Schweigg. [18], in general, these plants are recognized as *Anthyllis vulneraria* subsp. *maritima* (Hagen) Corb. [19]. However, molecular analysis did not reveal a clear genetic structure of *A. vulneraria* s. l. complex in Estonia, which would confirm intraspecies structure [20]. Also, on a wider European scale, no evidence for recognition of intraspecific taxa has been found [20]. Instead, phenological and reproductive traits within a species were strongly affected by both altitudinal and latitudinal gradients [21]. Therefore, high phenotypic plasticity might be a reason for the existence of significant morphological variations found between different local populations [22]. However, the existence of local genetic adaptation cannot be ruled out [23].

A. vulneraria plants from a population natively growing on heavy metal-enriched mine spoil heaps were shown to possess extreme metal tolerance together with a high accumulation capacity for zinc (Zn), lead (Pb), and cadmium (Cd) in aerial parts [24]. Since the potential for genetic diversity in a species is high, it is possible that geographically isolated populations of *A. vulneraria* exhibit different adaptive characteristics, including these related to heavy metal tolerance and accumulation. Plants of *Anthyllis vulneraria* subsp. *maritima*, found both on semi-fixed and fixed sand dunes, seem to be well adapted to conditions of moderate sand accretion [25].

Increased production of reactive oxygen species is one of consequences of deleterious heavy metal impact on plants [26]. Therefore, upregulation of the enzymatic antioxidative system plays an important part in plant heavy metal tolerance [1,27]. Two enzymes of the oxidative metabolism, peroxidase and polyphenol oxidase, are often used for the characterization of plant responses to deleterious abiotic conditions or biotic interactions [28,29]. In particular, the increase in leaf peroxidase activity in *A. vulneraria* subsp. *maritima* was a reliable indicator of rhizobial inoculation in plants [25]. In the context of heavy metal

tolerance, peroxidase is involved in several defense-related responses, including protection against ROS and the formation of impermeable barriers through lignification [30,31].

The aim of the present study was to explore heavy metal tolerance and accumulation potential in *A. vulneraria* subsp. *maritima* plants from coastal dunes in controlled conditions. It was hypothesized that the coastal-specific type of the species will have relatively good tolerance to metals in further confirming the view of the existence of species-wide metal tolerance of *A. vulneraria*. It was especially investigated if oxidative enzyme activity can be used as an indicator of heavy metal tolerance.

2. Results

Growth of *A. v. maritima* plants was differentially affected by various heavy metals used in the present study (Figure S1). The number of leaves, their biomass, and the biomass of the roots were negatively affected by increasing concentrations of Pb and Mn in substrate, but no negative effect was evident for Cd, Cu, and Zn (Figure 1). Root growth was stimulated by 20 mg L⁻¹ Cd and 100 mg L⁻¹ Cu treatments, but the effect was statistically significant only for Cu-treated plants (Figure 1C). Visible effects of metal toxicity were evident for Pb-treated plants (appearance of thinner leaves, yellowing of older leaves; Figure S1B), as well as for Mn-treated plants (reduced leaf size, curled leaves, red leaf venation; Figures S1D and S2). The relative distribution of the number of leaves in different leaf age groups, as well as biomass distribution among leaves of different age groups, was affected by heavy metal treatments (Figure 2). In all the treatments, the number and biomass of the middle leaves increased but these for young leaves decreased. An especially pronounced effect was evident for the Mn-treated plants. In plants treated with 500 and 1000 mg L⁻¹ Mn, the relative biomass of the older leaves also increased (Figure 2B). There was a significant decrease in water content in old leaves with increased Pb (500 mg L⁻¹) and Mn (200, 500, 1000 mg L⁻¹), indicating accelerated leaf senescence (Figure 3A). A significant decrease in root water content was evident only for plants treated with 1000 mg L⁻¹ either Pb or Mn (Figure 3B).

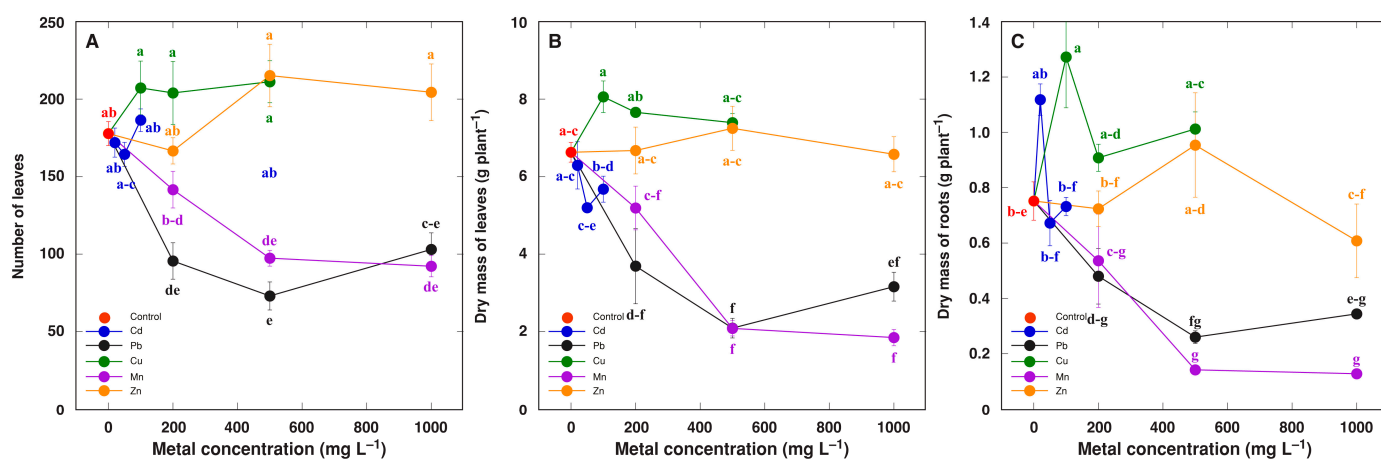


Figure 1. Effect of increasing concentration of heavy metals in substrate on number of leaves (A), dry mass of leaves (B), and dry mass of roots (C) of *Anthyllis vulneraria* subsp. *maritima* plants. Data are means ± SE from five replicates. Different letters of corresponding color indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

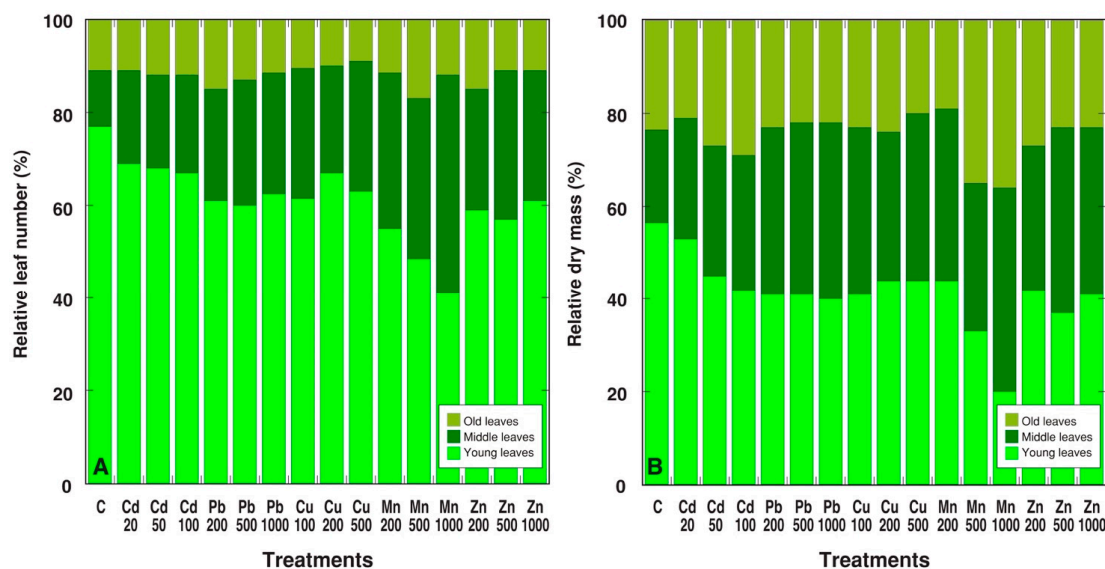


Figure 2. Effect of increasing concentration of heavy metals in substrate on relative distribution of number of leaves (A) and leaf dry mass (B) in different age groups of *Anthyllis vulneraria* subsp. *maritima* plants.

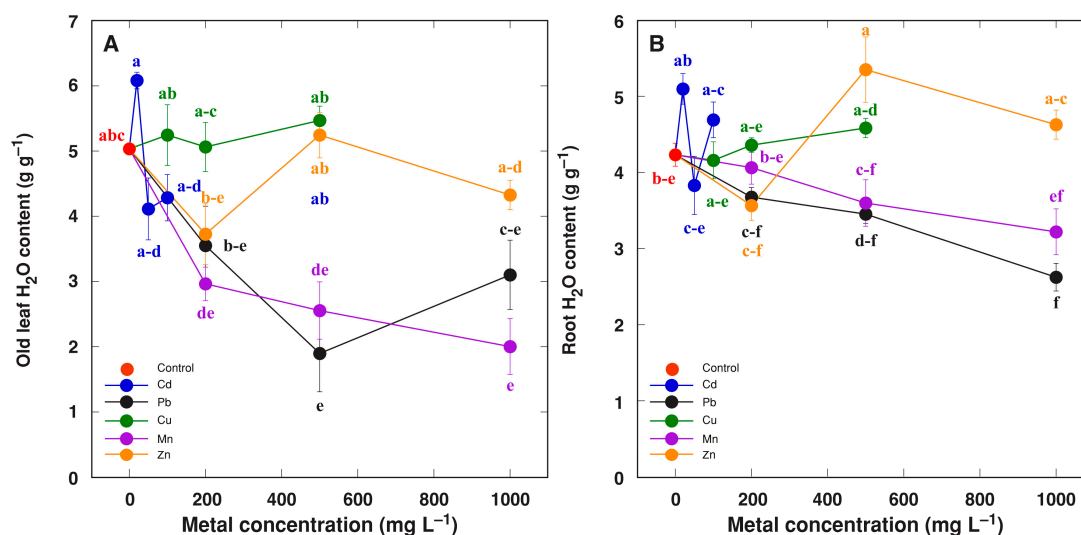


Figure 3. Effect of increasing concentration of heavy metals in substrate on water content in old leaves (A) and roots (B) of *Anthyllis vulneraria* subsp. *maritima* plants. Data are means $\pm$ SE from five replicates. Different letters of corresponding color indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

There was a characteristic increase in polyphenol oxidase activity in leaves of all the *A. v. maritima* plants treated with heavy metals (Figure 4A). The effect was significant starting from 100 mg L⁻¹ Cd, 500 mg L⁻¹ Pb, 200 mg L⁻¹ Mn, and 500 mg L⁻¹ Zn, but not for Cu-treated plants. In contrast, statistically significant increase in peroxidase activity was evident only for plants treated with 50 and 100 mg L⁻¹ Cd, 500 mg L⁻¹ Pb, 200–1000 mg L⁻¹ Mn, and 500 mg L⁻¹ Zn (Figure 4B).

Metal accumulation potential for Cd and Cu was the highest in the roots, but for Pb, Mn, and Zn, more metal accumulated in the old leaves (Figure 5). Accumulation of all the metals showed a typical saturation response to increasing substrate metal concentration, except Cd in the roots, Pb in the middle leaves, Cu in the old leaves, and Mn in the middle leaves and roots. For all the metals, accumulation potential was higher in the old leaves in comparison to that in the middle leaves.

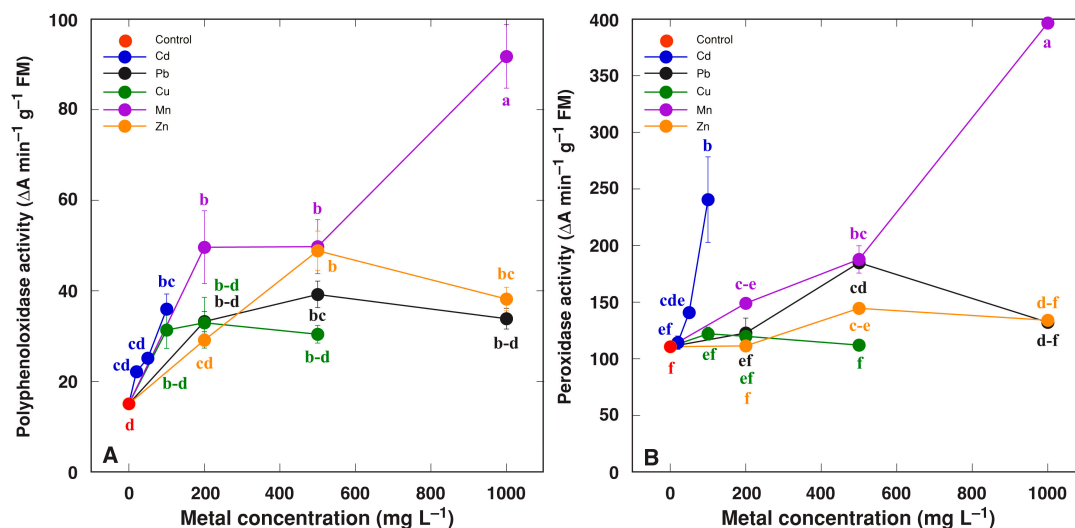


Figure 4. Effect of increasing concentration of heavy metals in substrate on polyphenol oxidase activity (A) and peroxidase activity (B) in old and middle leaves of *Anthyllis vulneraria* subsp. *maritima* plants. Data are means $\pm$ SE from six replicates. Different letters of corresponding color indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

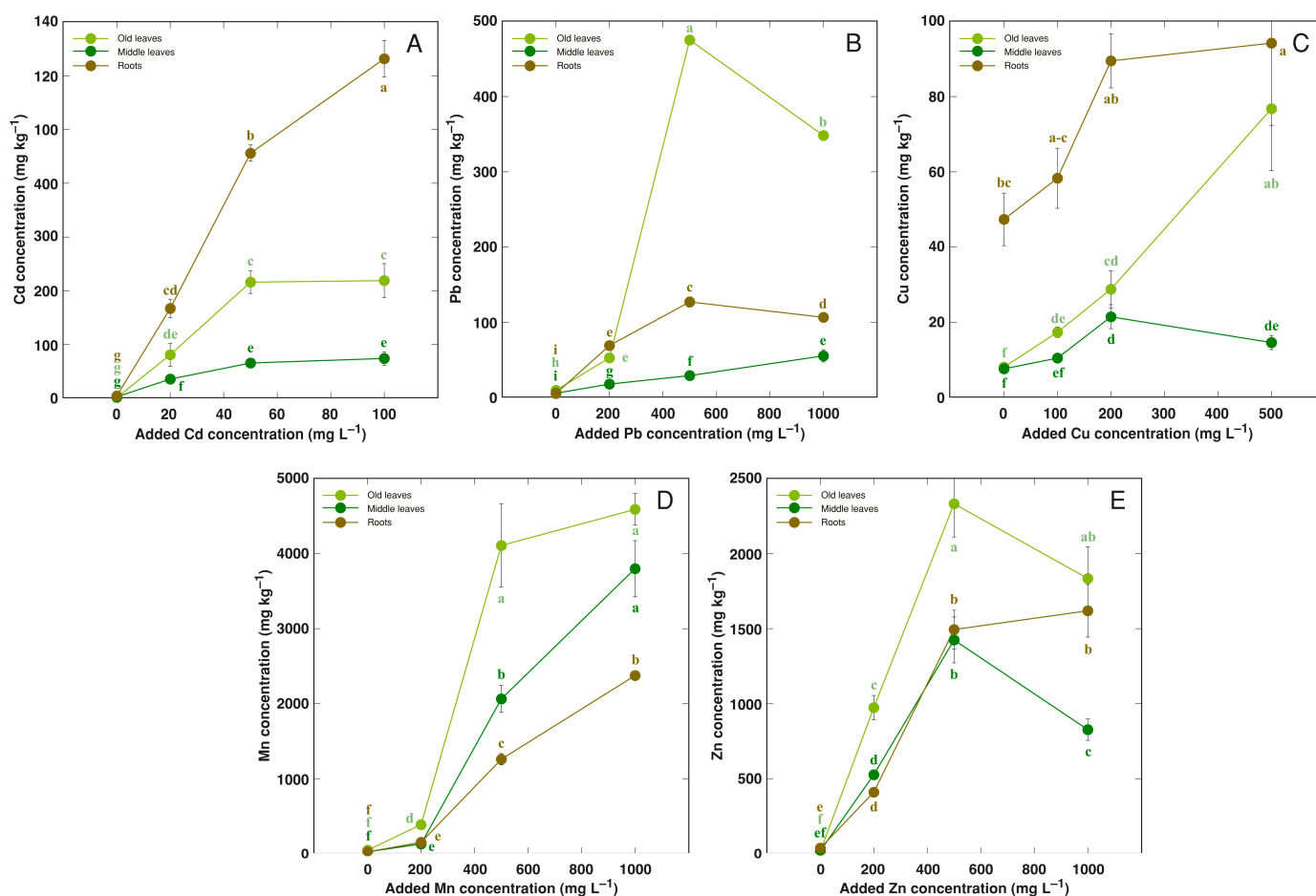


Figure 5. Effect of increasing concentration of heavy metals in substrate on accumulation of Cd (A), Pb (B), Cu (C), Mn (D), and Zn (E) in different parts of *Anthyllis vulneraria* subsp. *maritima* plants. Data are means $\pm$ SE from three replicates. Different letters of corresponding color indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$).

3. Discussion

3.1. Tolerance to Heavy Metals

A. vulneraria can be characterized as a facultative metallophyte species, as plants from metallicolous population had twice as high shoot mass when cultivated in unpolluted soil in comparison to that in mine tailing soil [32]. In the present study, *A. v. maritima* plants from coastal sand dunes showed relatively good but variable tolerance to heavy metals, indicating that metal tolerance might be a species-wide characteristic of *A. vulneraria*. So far, it was suggested that only *A. vulneraria* plants originating from highly polluted mine sites were able to grow on highly contaminated multi-metallic soil, and even in that case severe growth reduction occurred [32]. However, extreme substrate concentrations of Zn (35,000 mg kg⁻¹) and Pb (25,300 mg kg⁻¹) were used in that study. No comparative studies so far have been performed with soil-grown *A. vulneraria* plants from different populations in controlled conditions using a gradient of more realistic metal concentrations characteristic of contaminated soils, although, population-dependent growth responses of *A. vulneraria* plants have been found in conditions of hydroponics [33].

Even high concentrations of Cd, Cu, and Zn in substrate did not result in a growth reduction in *A. v. maritima* plants (Figure 1). However, treatment with all the metals resulted in a relative decrease in the proportion of young leaves in plant biomass (Figure 2). It can be suggested that the tolerance to Cd, Cu, and Zn in this experiment is provided by the efficient operation of internal protection systems, which include both protection against endogenous oxidative stress [34] and metal detoxification by chelation and their compartmentation [35,36].

Among all tested heavy metals, *A. v. maritima* plants were especially sensitive to increasing substrate Pb and Mn concentration. Treatment with either of the metals resulted in severe growth reduction (lower number of leaves, decrease in both leaf and root biomass), but under Mn toxicity leaf morphology was also negatively affected, which appeared as leaf curling, a reduction in leaf elongation, and red leaf venation. It seems that Mn tolerance is higher for plants native to flooding-prone habitats, where high concentration of plant-available Mn is a characteristic phenomenon [37]. Thus, salt marsh plants are shown to be relatively tolerant to high Mn content [38]. In addition, wetland plants with characteristic appearance in coastal habitats also show pronounced Mn tolerance, as *Ranunculus sceleratus* [39] and *Rumex hydrolapathum* [40].

The problem of the tolerance of legume species to abiotic factors needs to be solved in the context of their characteristic symbiosis with nitrogen-fixing bacteria. It has been established that the presence of bacterial symbiosis and the genotype of symbionts can change the tolerance of legume plants to various environmental factors [41–43]. In respect to *A. v. maritima*, it was shown that the presence of active rhizobial symbiosis modulated plant response to sand accretion [25]. In the current study, the formation of symbiotic relationship with nitrogen-fixing bacteria was not controlled, but, as the presence of nodules on the roots was not evident, it seems that the plants were non-symbiotic during the experiment.

It is clear that legume species from metal-contaminated soils also require metal-tolerant symbionts. Indeed, metal-tolerant species of nodule forming symbiotic bacteria was found in roots of *A. vulneraria* plants growing on mine tailings [44]. Initially, it was suggested that these bacteria are involved in the metal tolerance of the plants. However, the presence of symbiotic rhizobia had no effect on the heavy metal tolerance of *A. vulneraria* plants from a metallicolous population, but the accumulation of Zn, Pb, and Cd decreased in symbiotic plants irrespective of the type of rhizobia [45].

3.2. Metal Accumulation Potential

A. vulneraria plants naturally growing in calamine soil (10,000 mg kg⁻¹ Zn, 1562 mg kg⁻¹ Pb, 129 mg kg⁻¹ Cd) accumulated 2115 and 3858 mg kg⁻¹ Zn, 76 and 252 mg kg⁻¹ Pb, and 19 and 60 mg kg⁻¹ Cd, in shoots and roots, respectively [46]. Therefore, preferential accumulation of Zn is the most studied characteristic of *A. vulneraria* plants. *A. vulneraria* plants naturally growing on zinc mine deposits with only 733 mg kg⁻¹ of plant-available Zn accumulated up to 4400 and 5000 mg kg⁻¹ Zn in leaves and roots, respectively [10].

Maximum Zn accumulation capacity (16,000 mg kg⁻¹) was observed with *A. vulneraria* seedlings from a metallicolous population when cultivated in field conditions using tailing pond soil, containing 13,000 mg kg⁻¹ of Zn [47]. When the concentration threshold for Zn hyperaccumulation was reached, *A. vulneraria* plants were defined as Zn hyperaccumulators. When plant transplants from both metallicolous and non-metallicolous populations were cultivated in soils with various heavy metal content, it appeared that plants formed a metallicolous population that accumulated up to 4500–5000 mg kg⁻¹ Zn in the shoots, and those from non-metallicolous populations accumulated 2500–3000 mg kg⁻¹ Zn, but these differences leveled off with time [45]. In comparison, more than 2000 and 1500 mg kg⁻¹ Zn accumulated in old leaves and roots, respectively, of *A. v. maritima* plants from coastal sand dunes in the present study (Figure 5E).

Predominant accumulation of Zn in the leaves of *A. v. maritima* plants points to the presence of mechanisms similar to those of native hyperaccumulators. Both complexation with ligands and efficient compartmentation through specific transport mechanisms seem to be relevant mechanisms for high Zn accumulation in shoots. Thus, complex formation with small ligands, such as nicotianamide, is the main mechanism for Zn transport in hyperaccumulating species [48]. Vacuoles of leaf epidermal cells are final storage sites in hyperaccumulating species for several metals, including Zn [36,49].

A. vulneraria plants originating from seeds of the mine tailing population accumulated 278 and 1236 mg kg⁻¹ Cd in leaves and roots, respectively, when cultivated in hydroponics in the presence of symbiotic *Meserhizobium metallidurans*, but only 108 and 688 mg kg⁻¹ in leaves and roots, respectively, when cultivated in soil [50]. In the present study, even lower Cd accumulation potential was evident for soil-grown *A. v. maritima* plants, reaching 40 mg kg⁻¹ in older leaves (Figure 5A). Sulfur-containing ligands, such as glutathione and phytochelatines, are the most probable candidates for Cd complexation in plants, but other compounds also might be involved [35].

Information on Pb accumulation capacity in *A. vulneraria* appears to be very limited. Apart from a study using plants naturally growing on highly contaminated substrate, mentioned above [46], only one study measured Pb accumulation in tissue culture conditions. There, explants of *A. vulneraria* from a calamine soil had relatively low Pb accumulation potential (5 and 50 mg kg⁻¹, in leaves and roots, respectively) [51]. This is similar to the results obtained with *Armeria maritima*, where only 10–25 mg kg⁻¹ were accumulated in shoot explants in conditions of tissue culture, as compared to 200–400 mg kg⁻¹ in the leaves of soil-grown plants [52]. In the present study, *A. v. maritima* plants accumulated comparable tissue concentrations of Pb that reached 500 mg kg⁻¹ in old leaves (Figure 5B), which is comparably high level [53]. Translocation of Pb from roots to shoots is largely limited by lignified root endodermal cells [54], but complex formation with inorganic anions can be important for Pb accumulation in leaves associated with compartmentation in cell vacuoles [55].

Accumulation potential for Cu and Mn had not been evaluated previously in *A. vulneraria* plants. Accumulation potential for Cu can be characterized only as moderate, reaching 100 mg kg⁻¹ in roots and 80 mg kg⁻¹ in old leaves (Figure 5C). For comparison, *Armeria maritima* plants from a dry coastal meadow accumulated 200–400 mg kg⁻¹ Cu in roots and

450–620 mg kg^{−1} in older leaves [52]. Transport and detoxification of Cu is dependent on complexation with both small ligands (histidine and proline) and metallothioneins [56,57].

While Mn concentration in old leaves reached 4500 mg kg^{−1} (Figure 5D), this was associated with significant decrease in shoot biomass (Figure 1B) and other visual symptoms of Mn toxicity (Figure S2). It has been shown that coastal plant species usually accumulate relatively high amounts of Mn. Thus, *Hylotelephium maximum* plants accumulated up to 15,000 mg kg^{−1} Mn in leaves [58], *Armeria maritima* plants accumulated 4000–12,000 mg kg^{−1} Mn in older leaves [52], *Ranunculus sceleratus* accumulated 7500 mg kg^{−1} Mn in rosette leaves [39], and *Rumex hydrolapathum* accumulated 5500–6500 mg kg^{−1} Mn in leaves [40]. As the hyperaccumulation threshold for Mn has been set at 10,000 mg kg^{−1} [59], it is evident that many coastal species both from relatively dry (*Anthyllis vulneraria* subsp. *maritima*, *Armeria maritima*, *Hylotelephium maximum*) and wet habitats (*Ranunculus sceleratus*, *Rumex hydrolapathum*) have very high accumulation potential for this metal. In other Mn-accumulating plants, oxalic acid has been identified as a ligand for this metal [60]. Both photosynthetic and non-photosynthetic shoot tissues can act as sites of Mn deposition in different species with high Mn accumulation capacity [61].

Symbiosis with nitrogen-fixing bacteria is known to affect metal accumulation potential in legume species. For example, without nodulating bacteria, maximum accumulation potential in shoots was somehow higher than with active symbiosis, reaching 6229, 884, and 43 mg kg^{−1} for Zn, Pb, and Cd, respectively [32]. However, this was achieved at extremely high substrate heavy metal concentrations, reaching 35,000, 25,300, and 17 mg kg^{−1} for Zn, Pb, and Cd, respectively. A negative effect of symbiotic bacteria *Mesorhizobium metallidurans* on Zn accumulation capacity was noted also in an experiment performed with hydroponically cultivated plants from different populations [33]. However, in the presence of another bacterial symbiont, *Rhizobium metallidurans*, Zn concentration in the shoots increased by 36% [47]. Thus, it is highly possible that the properties of the symbiont significantly affect the metal accumulation potential of the plant and additional studies should be carried out with different bacterial isolates. The potential of legumes in phytoremediation, and, especially, rhizobium-improved phytoremediation potential of these species has become more widely recognized [62–64].

For *A. v. maritima* plants, hyperaccumulation threshold concentration values for the studied metals [9] were exceeded only for Cd, reaching more than 200 mg kg^{−1} in old leaves (Figure 5). However, the accumulation potential for Pb, Mn, and Zn in leaves was also extremely high, further suggesting excellent possibilities of using the species for practical application in the field of phytoremediation. From a fundamental biology perspective, the differences in the distribution of various metals between plant organs and the level of their accumulation are not entirely clear. Most likely, an ability to express metal-specific transmembrane transport protein genes underlies the observed accumulation differences between different metals [7,8].

3.3. Changes in Oxidative Enzyme Activity

Changes in the activity of oxidative enzymes have been frequently used as indicators of stress tolerance in different plant species [65–70]. Changes in peroxidase activity were not strongly expressed for plants treated with Cu and Zn, against which the plants were relatively tolerant (Figure 3B). However, in the case of Cd, against which the plants were also tolerant, peroxidase activity increased with increasing metal concentration, and the same was evident for Mn-treated plants, although they showed visible symptoms of toxicity. These differences reflect metal-specific effects of plant response and can be caused by different mechanisms. The treatment of pea plants with non-toxic Cd concentrations resulted in a relatively high increase in leaf peroxidase activity, but such an effect was

not evident in plants treated with toxic Cd levels [71]. For rice plants, it was shown that peroxidase represents a key enzyme in adaptation to Cd stress, directly or indirectly affecting Cd distribution [72]. Involvement of peroxidase in defense against ROS could be another explanation for the increase in enzyme activity, as Cd is known to induce the formation of ROS [73]. In the case of Mn toxicity, apoplastic leaf peroxidases have been shown to represent key enzymes for cowpea plants [74]. In particular, only a Mn-sensitive cultivar of cowpea showed increased peroxidase activity, which was associated with NADPH-dependent peroxidase species producing H_2O_2 in the apoplast.

It is also possible that, for heavy metals, the increase in peroxidase activity rather reflects the intensity of responses associated with defense mechanisms. Thus, peroxidase activity linearly increased in parallel with the accumulation of Cu, Pb, and Zn in leaves of relatively metal-tolerant species, *Avicennia marina* [75]. Most likely, peroxidase activity is nonspecifically induced as a result of metabolism activation in the case of induced defense responses. More specifically, different forms of peroxidases are involved in lignification or defense against ROS [30,31].

Our previous study with *A. v. maritima* plants showed that only an increase in peroxidase but not polyphenol oxidase activity in leaves was a good indicator of rhizobial inoculation [25]. However, sand burial increased leaf peroxidase activity only for non-inoculated plants. In another study with a wetland species *Rumex hydrolapathum* under a gradual Mn stress, a step-wise increase in peroxidase activity after each treatment indicated metal-induced physiological acclimation to Mn [76]. In the present study, a nonspecific increase in leaf polyphenol oxidase activity was evident for all metal treatments, but a specific high increase was evident only for plants treated with 1000 mg L^{-1} Mn (Figure 3A). In respect to polyphenol oxidase, it is suggested that the absence of an increase in the soluble activity of the enzyme under unfavorable conditions is an indicator of the stability of membranes and general plant tolerance, since, at least in some species, plastid polyphenol oxidase is tightly bound to membranes and is released only as a result of their integrity loss [66,77].

4. Materials and Methods

Seeds of *Anthyllis vulneraria* subsp. *maritima* (*A. v. maritima*) were collected at the end of August 2016 from plants growing on coastal sand dunes near Užava, Latvia. Seeds were stored at $4\text{ }^{\circ}\text{C}$. Experiments were performed during the winter season 2023–2024 in an automated experimental greenhouse. Details on initial plant establishment can be found elsewhere [25]. Briefly, seeds stored at $4\text{ }^{\circ}\text{C}$ were surface sterilized with diluted household bleach, scarified, and imbibed in sterilized deionized water. Seeds were further germinated in containers with autoclaved garden soil (Biolan, Eura, Finland) at $23\text{ }^{\circ}\text{C}$ until the appearance of the first two true leaves. Individual seedlings were successively transplanted to 200 mL containers with autoclaved soil for two weeks and then to 1.2 L plastic containers containing a mixture (3:2, *v/v*) of garden soil and quartz sand (Saulkalne-S, Saulkalne, Latvia).

Plants were cultivated in a greenhouse (HortiMaX, Maasdijk, The Netherlands) with supplemented light from Master SON-TPIA Green Power CG T 400 W (Philips, Amsterdam, The Netherlands) and Powerstar HQI-BT 400 W/D PRO (Osram, Munich, Germany) lamps ($380\text{ mol m}^{-2}\text{ s}^{-1}$ at the plant level), for a 16 h photoperiod, at day/night temperature $23/16\text{ }^{\circ}\text{C}$, and a relative air humidity of 60 to 70%. One week after the final transplanting, plants were randomly assigned to 16 treatment groups, with five replicates per treatment (Table 1). The metals selected for treatment and their concentrations were chosen according to the results of previous studies with other coastal plant species [39,40,52,58,78]. Treatment with heavy metals were performed gradually within two weeks, starting with the lowest

dose. The necessary amount of respective salt was dissolved in deionized water and 0.1 L per container was evenly applied to the soil surface in each container. One week after the last treatment, the plants were fertilized with Yara Tera Kristalon Red and Yara Tera Calcinit fertilizers (Yara International, Oslo, Norway). A stock solution was prepared for each fertilizer (100 g L^{-1}) and the working solution contained 25 mL of each per 10 L deionized water, used with a rate of 0.1 L per container. The substrate water content was monitored with a HH2 moisture meter equipped with a WET-2 sensor (Delta-T Devices, Burwell, UK) and kept at 50 to 60%.

Table 1. Treatments used in the present study.

Treatment (mg L^{-1} of Metal)	Salt	Total Amount of Salt (g per L of Soil)	Amount of Salt (g per L of Soil)		
			1st Treatment	2nd Treatment	3rd Treatment
Control	–	–	–	–	–
Cd 20	$\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$	0.041	0.041	–	–
Cd 50	$\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$	0.103	0.041	0.062	–
Cd 100	$\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$	0.205	0.041	0.062	0.102
Pb 200	$\text{PbOAc} \cdot 3\text{H}_2\text{O}$	0.366	0.366	–	–
Pb 500	$\text{PbOAc} \cdot 3\text{H}_2\text{O}$	0.916	0.366	0.550	–
Pb 1000	$\text{PbOAc} \cdot 3\text{H}_2\text{O}$	1.832	0.366	0.550	0.916
Cu 100	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.390	0.390	–	–
Cu 200	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.780	0.390	0.390	–
Cu 500	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.950	0.390	0.390	1.170
Mn 200	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.600	0.600	–	–
Mn 500	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.500	0.600	0.900	–
Mn 1000	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	3.000	0.600	0.900	1.500
Zn 200	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.880	0.880	–	–
Zn 500	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2.200	0.880	1.320	–
Zn 1000	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	4.400	0.880	1.320	2.200

Plants were cultivated for 4 weeks after reaching full treatment and then the experiment was terminated. At harvest, the plant leaves were separated according to their age, position, and size as old, middle, and young. Leaves were counted and weighed separately. Fresh leaf samples of old and middle leaves (one leaf of each per plant) were collected for enzyme analysis, quickly frozen in liquid nitrogen, and stored at -20°C . The roots were separated from the substrate and washed to remove any soil particles. The presence of nodules on the roots was not seen. After measurement of the fresh mass, tissues were dried at 60°C for 72 h and dry mass was measured. The tissue water content was estimated as a mass of water in grams per gram of dry mass.

Concentrations of Cd, copper (Cu), Pb, manganese (Mn), and Zn were measured separately in old leaves, middle leaves, and roots from three individual plants using microwave plasma atomic emission spectrometry (4200 MP-AES, Agilent, Santa Clara, CA, USA) as described previously [52].

Activities of peroxidase and polyphenol oxidase were measured as described previously [25]. Plant material was ground to a fine powder with a mortar and pestle using liquid nitrogen. The material was extracted for 15 min with 25 mM HEPES/KOH buffer (pH 7.2), containing 3% polyvinylpyrrolidone, 1 mM EDTA, and 0.8% Triton X-100. Af-

ter centrifugation ($15,000 \times g$ at 4°C , 20 min), supernatant was used for spectrophotometric measurement of peroxidase (with guaiacol and H_2O_2 at 470 nm) and polyphenol oxidase (with pyrocatechol at 410 nm) activity.

Results were analyzed by KaleidaGraph (v. 5.0, Synergy Software, Reading, PA, USA). The statistical significance of differences was evaluated by one-way ANOVA using post hoc analysis with an honestly significant difference. Significant differences were indicated by $p < 0.05$.

5. Conclusions

The study showed that the *Anthyllis vulneraria* genotype used in experiments is relatively highly tolerant to several heavy metals, especially Cd, Cu, and Zn. As the particular population grows naturally in coastal dunes where the substrate is free of elevated metal content, the obtained results show that the species has a general metal tolerance potential. On the other hand, the accumulation of several metals (Pb, Mn, Zn) mostly in leaves confirms the phytoremediation potential of the plants. Although the plants in this experiment were grown in a non-symbiotic state, available scientific information suggests that the symbiosis with nitrogen-fixing bacteria has a very high potential role in both plant tolerance and metal accumulation potential. The obtained results on the different changes in the activity of oxidative enzymes under the influence of different metals indicate the possible participation of different protective mechanisms in ensuring the tolerance of metals. Based on the present results, it seems that oxidative enzyme activity cannot be unequivocally used as a specific indicator of metal tolerance. In addition, phytoremediation potential for Cd, Pb, Mn, and Zn of *Anthyllis vulneraria* subsp. *maritima* plants can be characterized as relatively high.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/stresses5010006/s1>. Figure S1A: Typical Cd-treated *Anyhyllis vulneraria* subsp. *maritima* plants 4 weeks after the full treatment; Figure S1B: Typical Pb-treated *Anyhyllis vulneraria* subsp. *maritima* plants 4 weeks after the full treatment; Figure S1C: Typical Cu-treated *Anyhyllis vulneraria* subsp. *maritima* plants 4 weeks after the full treatment; Figure S1D: Typical Mn-treated *Anyhyllis vulneraria* subsp. *maritima* plants 4 weeks after the full treatment; Figure S1E: Typical Zn-treated *Anyhyllis vulneraria* subsp. *maritima* plants 4 weeks after the full treatment; Figure S2: Typical Mn toxicity symptoms on *Anyhyllis vulneraria* subsp. *maritima* plants two weeks after the full treatment.

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