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Comparison of the Effects of Gradual and Acute Treatment with Mn on Physiological Responses of *Rumex hydrolapathum* Plants

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Abstract: An understudied problem in plant heavy metal biology is the effects of acute versus gradual or chronic metal exposure. The aim of the present study was to compare the growth and physiological responses of *Rumex hydrolapathum* Huds. plants subjected to gradual or acute Mn stress treatment in controlled conditions. Heavy metal was applied to substrate either as one 1.00 g L^{-1} Mn dose (acute treatment) or the same dose in four steps of increasing amounts within 12 days (gradual treatment). Peroxidase activity in actively photosynthesizing leaves was used for monitoring induced biochemical changes resulting from Mn treatment. The number of leaves per plant significantly increased in the case of gradual treatment with Mn, but this effect was not statistically significant for acute treatment. Leaf fresh mass significantly decreased in both cases due to the decrease in leaf water content, but dry biomass of leaves was not affected, with no significant differences between the two types of treatments. A significantly lower chlorophyll fluorescence parameter Performance Index in large leaves of plants under the acute Mn treatment than in plants under the gradual treatment was evident. An increase in leaf peroxidase activity by Mn treatment was proportional to the metal dose received, but plants in the acute treatment with 1.00 g L^{-1} Mn had a significantly lower peroxidase response in comparison to the gradual treatment with 1.00 g L^{-1} Mn. In conclusion, under gradual treatment, biochemical changes related to the induction of tolerance to the heavy metal are expressed, as indicated by the continuous increase in leaf peroxidase activity after each treatment step.

Keywords: chlorophyll fluorescence; electrolyte leakage; heavy metals; manganese; peroxidase; *Rumex hydrolapathum*

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1. Introduction

Studies on the toxicity of heavy metals in plants and the mechanisms ensuring their metal tolerance have become especially relevant in the conditions of unabated global environmental pollution and in connection with the possibility of using plants in environmental remediation technologies [1–3]. In contrast to most crops, which are relatively susceptible to high metal concentrations in the environment, studies of wild plants from soils with naturally high metal content or saline wetland species with special metal tolerance and accumulation potential are of particular interest [4–9].

An understudied problem in plant heavy metal biology is the effects of acute versus gradual or chronic metal exposure. It is well known that subtle changes in soil chemical composition rarely occur in nature, and plants are usually in a situation of low doses of heavy metals during relatively long time. However, in experiments in controlled conditions, plants are often exposed to subtle increase in high heavy metal concentration in a root medium, and significant variation in treatment techniques and regimes between different experiments significantly affect outcome of plant responses and can make generalization of the obtained information difficult. One important aspect related to acute treatment could be osmotic stress in the root environment due to a rapid increase in salt concentration, leading to immediate unfavorable physiological consequences similar to those during NaCl

treatment [10]. Differences in NaCl application either as gradual or single-step treatment really show a significant effect both at the level of gene expression, as well as metabolism and morphology [11]. Therefore, it is reasonable to predict that the type of application of heavy metals (gradual vs. single application) will significantly affect plant responses, especially when using relatively high metal concentrations.

Accelerated production of reactive oxygen species (ROS) is an inevitable consequence of heavy metal stress in plants, leading to both peroxidative damage to membranes and inactivation of antioxidative enzymes [12–14]. Therefore, both the constitutive and induced capacity of enzymatic antioxidative system are crucial components in heavy metal tolerance in plants [12–14]. Peroxidase activity, measured in plant tissue extracts by means of different phenolic type electron donors and hydrogen peroxide, is very often used as a general indicator of the capacity of the enzymatic antioxidative system [15]. However, physiological functions of peroxidases in plants are related not only to antioxidative system but also to variety of development-related processes [16–18]. Multiple molecular forms of peroxidase, being both under developmental and environmental control, can exist in single plant species, but specific functions of individual peroxidases are difficult to assign due to low specificity towards phenolic substrates as well as possible modifications at the protein level [19].

Mn is an essential micronutrient in plants, but can have toxic effects if accumulating in soil in plant-available forms, especially at low soil pH and decreased redox potential [20–22]. Poor aeration in wetland soils often lead to increased bioavailability and toxicity of Mn [23,24]. Moreover, the concentration of Mn in contaminated natural wetland sediments can reach as high as 1100 mg kg^{-1} [8]. Mn sensitivity is clearly a genotype-dependent feature, and many wetland species are shown to be highly tolerant to this metal, especially in conditions of salt marshes [25,26]. As Mn is a mobile element in plants, toxicity symptoms first appear on photosynthetically active mature leaves with a higher Mn accumulation capacity [27]. Therefore, the shoot growth of Mn sensitive plants is usually more negatively affected in comparison to that of roots [28]. The deleterious effects of Mn are clearly associated with an increase in ROS production [29,30]; therefore, the induced expression of antioxidative enzymatic system components is an important constituent of Mn tolerance [31–33]. In addition, genotypes more tolerant to Mn have a higher activity of peroxidase [34].

Several indicators of ROS-dependent heavy-metal-induced membrane lipid peroxidation have been used, including malondialdehyde (MDA) concentration (often measured as a total amount of thiobarbituric acid-reactive substances) and electrolyte leakage intensity from tissue samples. Usually, these two parameters show a parallel and proportional increase with the increase in the harmful effect of heavy metals [35]. In addition, a decrease in photosynthesis-related parameters is often used as an indicator of heavy-metal-associated physiological disorders, including those of Mn [36,37].

Several species of genus *Rumex* (Polygonaceae) have been characterized as heavy metal-tolerant species with a promising potential for metal accumulation [38–42]. *Rumex hydrolapathum* Huds. is a perennial high biomass-forming species naturally growing in wet and flooded habitats with a high disturbance frequency [43]. Tolerance to heavy metals, including Mn, in *R. hydrolapathum* has been mostly associated with physiological mechanisms, as the metals predominantly accumulated in older leaves, with appearance of characteristic visual signs of toxicity, followed by leaf senescence and dieback [44,45]. Along with it, formation of new leaves was stimulated. Thus, *R. hydrolapathum* can serve as a promising model species in studies of heavy metal tolerance mechanisms.

The aim of the present study was to compare the growth and physiological responses of *R. hydrolapathum* plants subjected to gradual or acute Mn stress treatment. As physiological indicators, photosynthesis-related parameters, electrolyte leakage and peroxidase activity were used. It was hypothesized that an acute treatment with Mn will have more pronounced or/and negative responses in comparison to that of gradual treatment.

2. Results

Treatment with $MnSO_4$ resulted in a significant increase in substrate electrical conductivity and a decrease in pH, but there were no significant differences in dependence of the treatment type (Figure 1). The number of leaves per plant significantly increased in the case of gradual treatment with Mn, but this effect was not statistically significant for the acute treatment (Figure 2). Leaf fresh mass significantly decreased in both cases due to the decrease in leaf water content, but dry biomass of leaves was not affected. Again, no significant differences were found between the two types of treatments. However, the number of old leaves and their dry biomass tended to increase more in plants gradually treated with Mn, in comparison to the control plants and these treated with only one dose of Mn (Figure 3). The proportion of large leaves in the total biomass decreased by Mn treatment, and this effect was more pronounced for plants under the gradual Mn treatment. Further, treatment with Mn tended to increase both the number and biomass of new leaves.

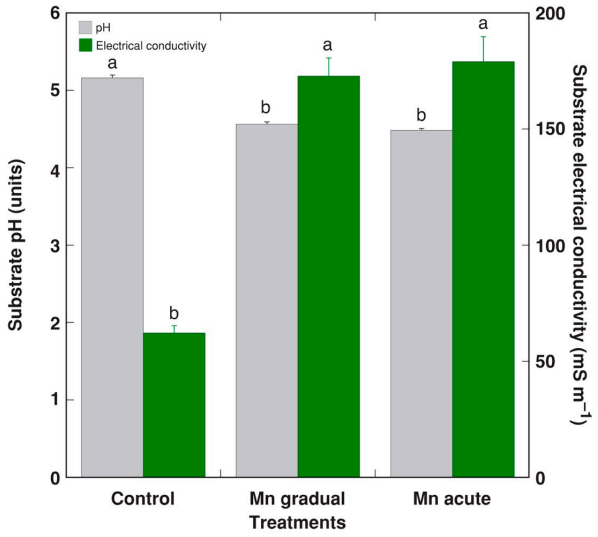

Figure 1. Effect of treatment type with Mn (1.00 g L^{-1}) on pH and electrical conductivity in substrate with *Rumex hydrolapathum* at the end of the experiment. Values are the means ± SE from five replicates with four independent measurements each. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$) for a particular parameter.

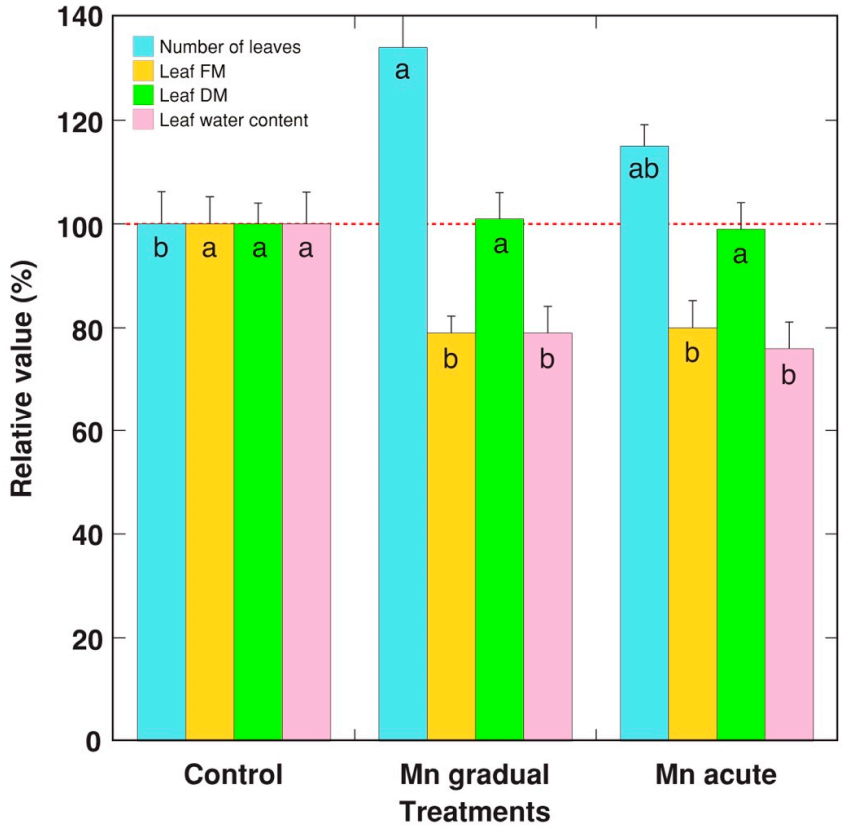

Figure 2. Relative effect of treatment type with Mn (1.00 g L^{-1}) on morphological parameters of *Rumex hydrolapathum*. Values are the means ± SE from five replicates. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$) for a particular parameter. FM, fresh mass; DM, dry mass. The dotted line indicates the control level.

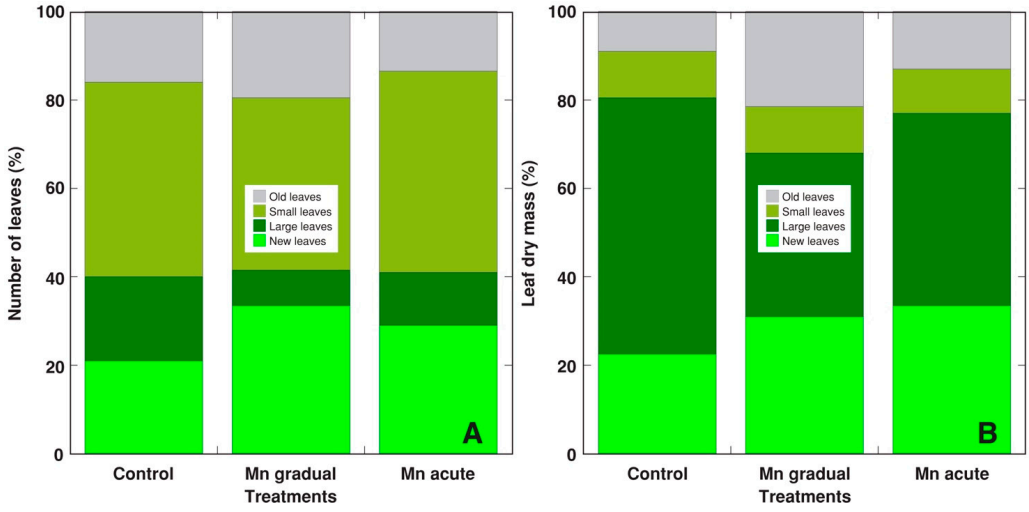

Figure 3. Relative distribution of the number of leaves (**A**) and dry leaf biomass (**B**) among different leaf classes of *Rumex hydrolapathum* plants differentially treated with Mn (1.00 g L^{-1}).

Chlorophyll concentration in large leaves did not significantly change by Mn treatments, but it was significantly lower in old leaves of plants under the acute Mn treatment in comparison to that of control and gradually Mn-treated plants (Figure 4A). Values of chlorophyll *a* fluorescence parameter Performance Index were lower in older leaves in comparison to these in large leaves (Figure 4B). A significantly lower Performance Index in large leaves of plants under acute Mn treatment than in plants under gradual treatment was

evident, but there was no statistically significant difference from control plants. Chlorophyll *a* fluorescence parameter F_v/F_0 tended to be lower in old leaves but showed no significant changes in dependence of either type of Mn treatment (Figure 4C). Intensity of electrolyte leakage was higher in old leaves, but no significant differences were evident for plants between the different treatments (Figure 4D).

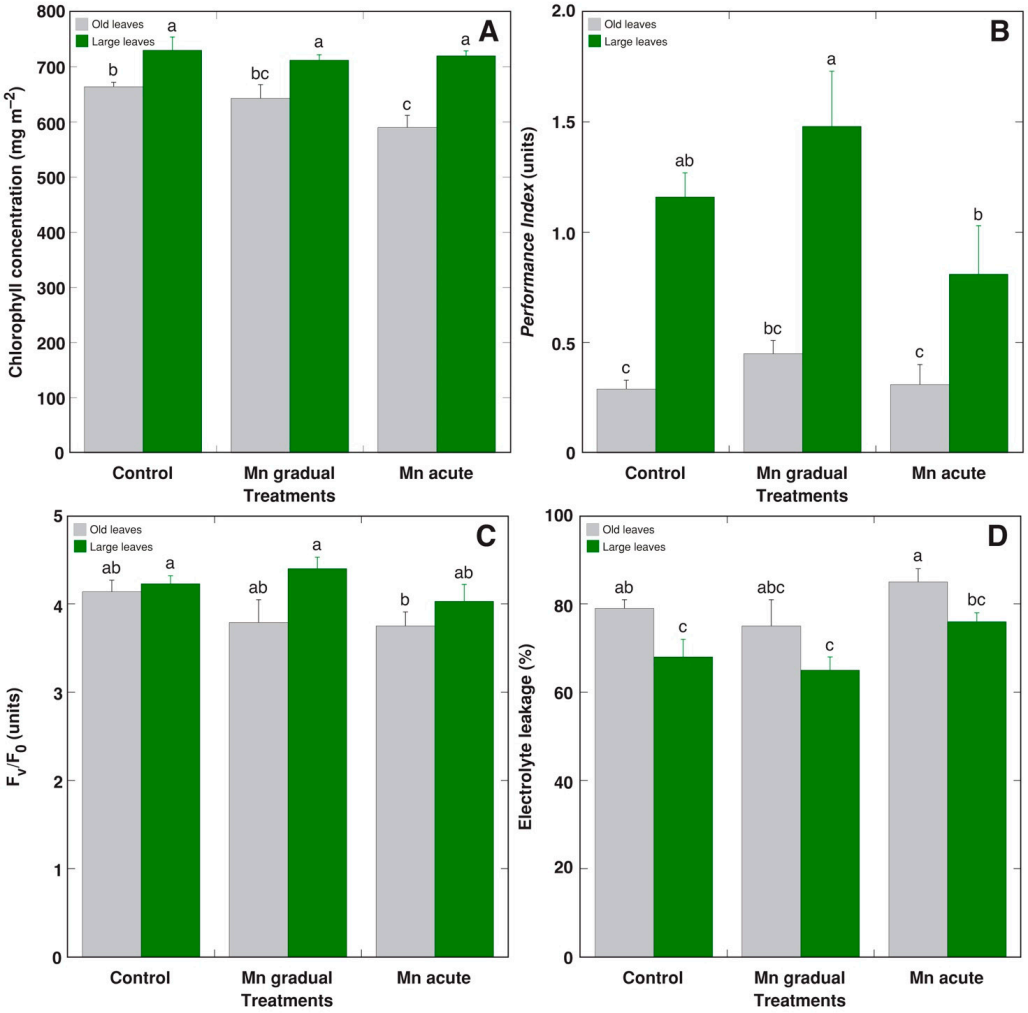

Figure 4. Effect of treatment type with Mn (1.00 g L^{-1}) on leaf chlorophyll concentration (**A**), Performance Index (**B**), F_v/F_0 (**C**), and electrolyte leakage (**D**) of *Rumex hydrolapathum*. Values are the means ± SE from 10 replicates for A, B, C and three replicates for D. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$) for a particular parameter.

Peroxidase activity in large leaves of *R. hydrolapathum* was measured periodically during the experiment in plants of six treatment groups (Figure 5). Peroxidase induction competence by Mn treatment was low for plants before day 13, as the activity in Mn-treated plants steady increased irrespective of treatment dose (Figure 5). After day 13, peroxidase activity increased in all plants previously or currently treated with Mn, but proportionally to the treatment dose. However, plants gradually treated with 0.50 and 1.00 g L^{-1} Mn showed identical response. While the absolute level of peroxidase activity in plants received acute treatment with 1.0 g L^{-1} Mn was lower than that in plants gradually treated with the same total dose of Mn, the relative increase in peroxidase activity after day 13 was relatively similar, showing a 4.5- and 3.5-fold increase over the previous peroxidase activity level, respectively.

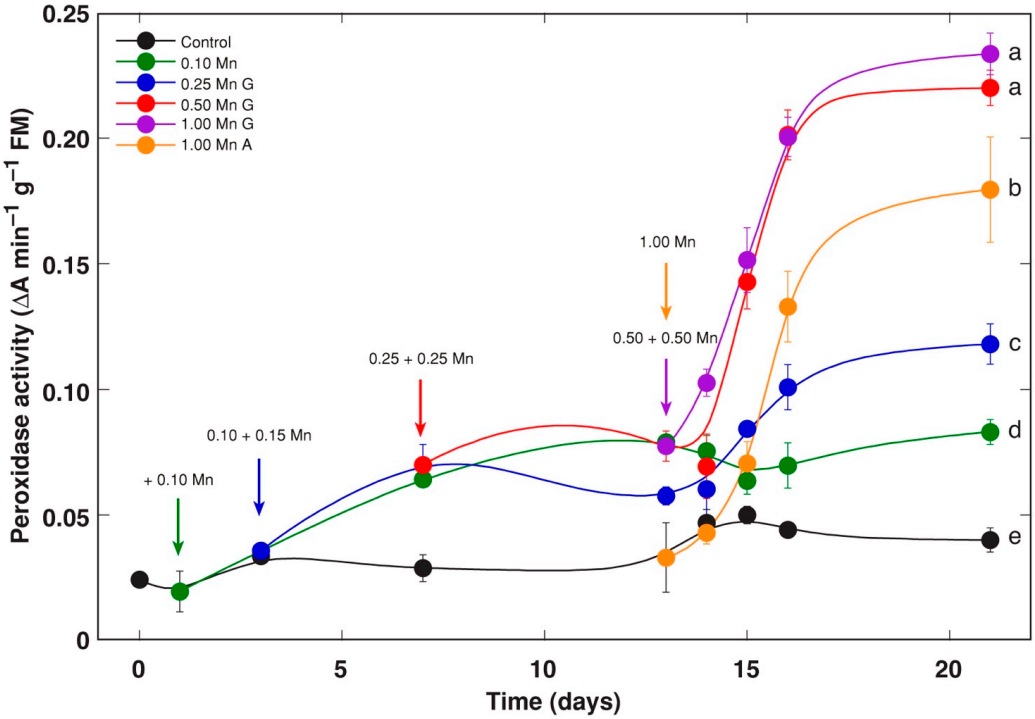

Figure 5. Time course of peroxidase activity in large leaves of *Rumex hydrolapathum* plants as affected by the type of treatment with Mn. Arrows of the respective color indicate time of treatment with particular doses of Mn in g per 1 L of substrate in addition to the already received doses. G, gradual; A, acute; FM, fresh mass. Values are the means ± SE from two biological replicates with four independent measurements each. Different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$) at the end of the experiment.

3. Discussion

In the present study, possible differences between gradual and acute treatment with heavy metals (Mn) at the level of growth responses as well as different physiological indicators (photosynthesis-related parameters, electrolyte leakage, peroxidase activity) of relatively metal-tolerant species *Rumex hydrolapathum* were evaluated. The obtained results have certain practical importance, as it is evident that gradual treatment with heavy metal can lead to acclimation of plants associated with multiple induction of peroxidase activity after each treatment step. The outcome of this study also has methodological importance, as one can argue that the type of treatment with heavy metals, including differences in the timing of treatment, affects results of the particular experiment.

One of the characteristic specificity of the model plant species is that related to morphological characteristics of rosette-forming plants with continuously active leaf-forming apical meristem, like in *R. hydrolapathum*, which allow for clearly distinctive adaptive strategy in the case of soil chemical contamination. The surplus concentration of essential mineral elements (as K, Mn and Zn) or bulk amount of any unnecessary ions (as Na and Cl) primarily accumulate in the older leaves, followed by their accelerated senescence and stimulation of new leaf formation [44–46]. This type of development can be considered as part of an avoidance strategy, since the accumulation of potentially toxic elements does not occur in actively photosynthesizing leaves. Further, biochemical protection against the presence of metals is also induced in actively photosynthesizing leaves, as shown by the observed increase in peroxidase activity in plants.

Among the limitations of this study, it needs to be stressed that the performed experiment was relatively short term, as *R. hydrolapathum* plants were harvested only one week after the acute treatment with Mn, but gradually treated plants received Mn within two weeks, followed by one more week before harvest. Although there was no effect of the

type of treatment on the morphological indicators, even in this situation, the physiological characteristics showed that acute treatment has a potentially more adverse effect compared to gradual treatment. Thus, faster senescence of older leaves of plants under acute Mn treatment was indicated by their lower water content and decreased chlorophyll concentration in comparison to (Figure 4A). In addition, chlorophyll *a* fluorescence parameter Performance Index was lower in plants under acute treatment in comparison to that in plants under gradual treatment (Figure 4B). However, the degree of membrane damage only tended to be higher in plants treated with one dose of Mn, as indicated by the values of electrolyte leakage (Figure 4D).

Among metabolic indicators of endogenous oxidative stress, changes in MDA concentration or electrolyte leakage capacity are most often considered. As heavy metal treatment results in increased production of ROS, usually changes in both parameters proportionally correlate in plants treated with increasing doses of heavy metals [47]. From a functional point of view, the question might arise if changes in peroxidase activity are related to heavy metal toxicity or plant tolerance to heavy metals? It is logical to assume that at low and physiologically tolerable concentrations of heavy metal, an increase in peroxidase activity represents a part of tissue tolerance mechanism to the metal in a form of upregulation of enzymatic antioxidative system. Further, when the heavy metal concentration exceeds some threshold value for toxicity for the particular plant species, a decrease in peroxidase activity with a further increase in the metal concentration could show the negative consequences of the general metabolic stress situation associated with the breakdown of control systems and the inability to regulate the expression of defense genes [48]. However, it is often seen from the provided experimental results that peroxidase activity and MDA concentration increase nearly linearly in parallel with increased heavy metal concentration, often leading to more negative consequences for plant growth [49]. According to that logic, if an efficient enzymatic antioxidative system activity is induced, an increase in MDA concentration or any other indicator of membrane damage should not be observed. Thus, in the present study, while electrolyte leakage capacity was higher in older leaves, it did not significantly change in leaves of Mn-treated plants (Figure 4D).

Recently, there has been no extensive comparative analysis of the possible functions of peroxidase in defense against heavy metals. However, peroxidase activity measurements are still being actively used in studies on the effects of heavy metals in plants [49–54]. The observed changes in peroxidase activity in leaves of *R. hydrolapathum* plants more likely reflect defense-related responses similar to these occurring during priming of induced tolerance [55,56]. In this context, the case of gradual heavy metal treatment can be considered as the manifestation of induced tolerance, where it is possible to increase plant survival and growth by treatment with another factor at small intensity or the potentially harmful factor itself at a small dose [57]. The phenomenon of priming in respect to the metal stress in plants has been analyzed in detail recently [58]. In a stepwise treatment, each individual treatment acts as an inducing factor, leading to changes in gene expression and metabolic adaptation to the next treatment with a higher dose, as evidenced by each increase in peroxidase activity in the present study.

It is also evident that different components of enzymatic antioxidative system have different sensitivity to endogenous oxidative stress. Thus, it can often be observed that peroxidase activity continues to increase while the activity of other enzymes of antioxidative protection decreases as the metal dose increases [59]. More specifically, for Mn, peroxidase activity on protein basis in both leaves and roots increased with increasing doses of Mn in soybean plants in spite of significant growth inhibition and visual toxicity symptoms [28]. In addition, peroxidase activity did not change as a result of growth-inhibiting dose of Mn in *Spirodela polyrhiza* plants, while catalase activity decreased significantly [36]. However, in *Polygonum hydropiper* plants, both peroxidase and catalase activity showed maximum levels at moderate Mn doses and decreased further [37]. Interestingly, apoplastic NADH-dependent peroxidase has been associated with appearance of visual toxicity symptoms in *Vigna unguiculata* plants at high Mn doses [60]. It is highly likely that compartmentalization

of peroxidase isoforms at both tissue and cellular level is an important factor in understanding the role of the enzyme in plant responses and tolerance to heavy metals. The relatively small but significant increase in peroxidase activity in control plants during cultivation could be related to increased lignification of plant leaf vasculature, leading to increased mechanical resistance of leaves in adult plants [19]. Further, increased lignification as a response to heavy metal treatment has been pointed out, which also could be associated with peroxidase functions [61–63].

In the present study, acute treatment with 1 g L^{-1} Mn was performed at the same time point when the summed dose of Mn in the gradual treatment also reached 1 g L^{-1}. This allowed for direct comparison of physiological responses in plants. However, due to these methodological features, the time period after the acute treatment until the termination of the experiment was relatively short, so the visual symptoms of acute toxicity could not appear. Also, morphological differences between the two types of treatment were not evident (Figure 2), while physiological indicators showed more severe stress situation in the case of acute treatment in comparison to the gradual one (Figure 4). Also, acute treatment with 1.00 g L^{-1} Mn had a significantly lower peroxidase response in comparison to the gradual treatment with 1.00 g L^{-1} Mn (Figure 5). Thus, it is possible to predict that growth inhibition will be more severe and visual toxicity symptoms will appear sooner in the case of acute treatment as compared to the gradual treatment with Mn.

It can be concluded that the treatment type has a significant effect on the physiological responses of *R. hydrolapathum* plants to biogenous heavy metal Mn. Under gradual treatment, biochemical changes related to the induction of tolerance to the heavy metal are expressed, as indicated by a continuous increase in leaf peroxidase activity after each treatment step. Future studies should include other treatment schemes to better assess possible differences in the growth and degree of visual damage as related to changes in biochemical indicators of heavy metal stress.

4. Materials and Methods

4.1. Plant Material and Establishment of Seedlings

Seeds of *Rumex hydrolapathum* Huds. Were collected from plants growing in a sea-affected wetland near Mersrags, Latvia at the beginning of September 2018. Seeds were kept for one month in laboratory conditions and further were stored at 4 °C. After imbibition in sterile deionized water for 2 h, seeds were placed 1 L plastic tissue culture containers containing autoclaved garden soil (Biolan, Eura, Finland) moisturized with sterile deionized water. Containers were incubated at photon flux density 40 µmol m^{-2} s^{-1} of photosynthetically active radiation with a 16 h photoperiod, day/night temperature 20/15 °C in a plant growth cabinet MLR-352H (Sanyo Electric, Osaka, Japan).

4.2. Plant Cultivation and Treatment Conditions

After the appearance of the first two true leaves, seedlings were individually transplanted to 200 mL plastic containers with a mixture of garden soil (Biolan, Eura, Finland) and quartz sand (Saulkalne S, Saulkalne, Latvia), 4:1 (*v*/*v*). Containers with plants were placed in 48 L closed plastic boxes and further gradually adapted to greenhouse conditions. An automated experimental greenhouse system (HortiMaX, Maasdijk, The Netherlands) was used for plant cultivation. A natural daylight was supplemented with photon flux density 380 µmol m^{-2} s^{-1} of photosynthetically active radiation with a 16 h photoperiod, provided by Powerstar HQI-BT 400 W/D PRO (Osram, Munich, Germany) and Master SON-TPIA Green Power CG T 400 W (Philips, Amsterdam, The Netherlands) lamps. The day/night temperatures were 23/16 °C, the relative air humidity 60–70%.

When the height of the seedlings reached 5–10 cm, they were individually transplanted to 1.2 L plastic containers filled with 1.0 L of the same soil and sand mixture as indicated previously. Substrate water content was monitored daily with an HH2 moisture meter equipped with a WET-2 sensor (Delta-T Devices, Burwell, UK) and a necessary amount

of deionized water was added to maintain 65–75% moisture. Individual containers with plants were randomly placed on greenhouse bench and repositioned twice a week.

One week after the final transplanting, 94 uniform plants were randomly distributed in six treatment groups (Figure 6). The large number of plants in this and other treatments was due to the need for destructive sampling for peroxidase analyzes during the experiment. In total, 26 plants were left untreated and served as control. A batch of 52 plants were treated with 0.10 g Mn in a form of $MnSO_4$ on day 1, followed by treatment of 38 plants from this batch with 0.15 g Mn on day 3. From these, 26 were treated with 0.25 g Mn on day 7. Further, 16 of these plants were treated with 0.50 g Mn on day 13. The remaining 16 plants were treated with 1.00 g Mn on day 13. For treatments, the required amount of salt was dissolved in 200 mL of deionized water and poured evenly on the soil in the container with the plant.

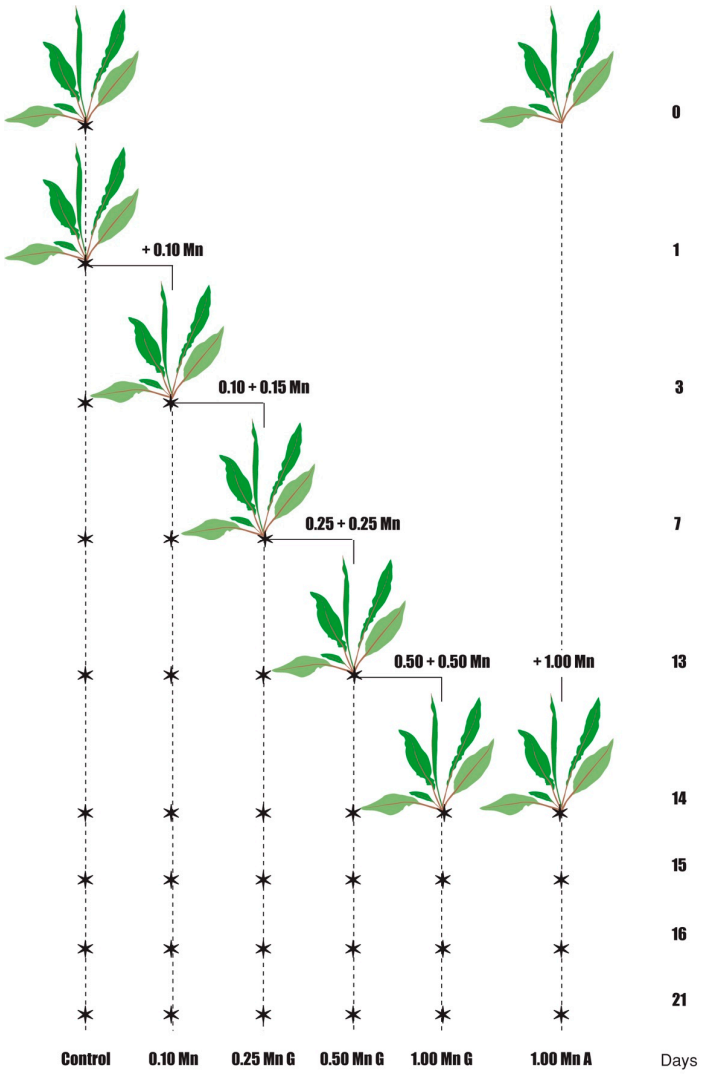

Figure 6. Schematic representation of performed treatments with Mn in a form of $MnSO_4$. Numbers indicate summary doses of Mn in g per 1 L of substrate. G, gradual; A, acute. Asterisks indicate leaf sampling for peroxidase analysis. Only control, 1.00 Mn G and 1.00 Mn A plants were sampled for morphological analysis at day 22.

4.3. Measurements and Experiment Termination

On the days marked with an asterisk (Figure 6), from two plants per treatment, the leaf of the longest plant at that time was collected for peroxidase activity analyses. Plant tissues were frozen in liquid nitrogen and stored at −20 °C until analysis. For measurement of

peroxidase activity, each collected leaf was longitudinally cut in half. Enzyme extraction was performed using 0.5 g tissue samples from halves of two leaves per treatment. The samples were ground to a fine powder with mortar and pestle, and extracted with 25 mM HEPES/KOH buffer (pH 7.2), containing 1 mM EDTA, 3% polyvinylpolypirrolidone, 0.8% Triton X-100. For each sample, 2.5 g buffer was used and extraction was performed for 15 min. After centrifugation at 15,000× *g* for 20 min at 4 °C, the supernatant was used for spectrophotometric measurement of enzyme activity. The reaction mixture contained 2 mL 50 mM $NaPO_4$ buffer (pH 7.0) with 10 mM guaiacol, 0.5 mL 0.03 mM H_2O_2 and 0.01 mL enzymatic extract. The mixture without H_2O_2 was used as a reference. The increase in optical density (OD) at 470 nm was followed for 3 min. Peroxidase activity was calculated as a rate of increase in OD from the linear portion of the OD curve over 30 s and expressed per g of fresh biomass.

Chlorophyll concentration and chlorophyll *a* fluorescence measurements were performed before the termination of the experiment using two older leaves and two larger leaves of each plant. Only plants from control, 1.00 Mn G and 1.00 Mn A treatments were used, with five plants per each treatment. Chlorophyll concentration in plant leaves was measured by a chlorophyll meter CCM-300 (Opti-Sciences, Hudson, NH, USA). Chlorophyll *a* fluorescence was measured in leaves that had been dark adapted for at least 20 min by a Handy PEA fluorometer (Hansatech Instruments, King's Lynn, UK). For characterization of photochemical activity, fluorescence parameters F_v/F_0 and Performance Index Total were used [64]. F_v/F_0, calculated as $(F_m - F_0)/F_0$, reflects an instant photochemical activity at the donor side of photosystem II. Performance Index Total is used as a relative indication of plant vitality, and includes information on the status of both photosystem II and photosystem I, in addition to characterizing the electron flow between the two systems, which is on an absorption basis.

Three plants per treatment (from control, 1.00 Mn G and 1.00 Mn A) were used for measurement of relative electrolyte leakage [65] using two older leaves and two larger leaves of each plant. A batch of 15 discs (0.5 cm^2) was prepared from fresh leaves, rinsed with deionized water three times in immersed in tubes with 10 mL deionized water for 22 h at room temperature. Initial electrical conductivity of the solution was measured using LAQUAtwin compact conductivity meter B-771 (Horiba Scientific, Kyoto, Japan). After incubation in a water bath at 80 °C for 2 h and cooling to room temperature, final electrical conductivity was measured. Relative electrolyte leakage was calculated as the difference between the final and the initial conductivity and expressed as the percent.

At the end of the experiment, substrate pH and electrical conductivity was measured in the containers with *R. hydrolapathum* plants. Substrate pH was measured using a pH meter pH 3000 (STEP Systems, Nürnberg, Germany). For measurement of electrical conductivity, a HH2 moisture meter equipped with a WET-2 sensor (Delta-T Devices, Burwell, UK) was used. Five plants per treatment were used. For every container, four separate readings on all sides of the container were performed for both parameters.

The experiment was terminated on day 22. Dry leaves was harvested separately, but living leaves were divided in old, small, large, and new leaves, according to their age an position, as well as size. Five plants per treatment (from control, 1.00 Mn G and 1.00 Mn A) were used for measurement of both fresh and dry (after drying in an oven at 60 °C for 72 h) biomass. Leaf water content was calculated as g H_2O per g of dry mass.

4.4. Data Analysis

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

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