

Article

Genotype-Specific Responses of *Anthyllis vulneraria* to Lead and Manganese Stress Mediated by Rhizobacterial Symbiosis

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Abstract

Legume–rhizobia symbiosis plays an important role in plant responses to stressful environments, including soils contaminated with heavy metals. *Anthyllis vulneraria*, a widespread wild legume, exhibits genotype-dependent variation in metal tolerance and accumulation. The objective of this study was to compare responses of three *A. vulneraria* genotypes to Pb and Mn stress and to test the hypothesis that tolerance is influenced by rhizobial inoculation with a specific strain. Plants were cultivated under partially controlled conditions with or without inoculation and subjected to Pb or Mn stress. Growth, nodulation, tissue water content, photochemical performance, and metal accumulation were assessed. Both metals negatively affected plant growth and physiological performance; however, responses were genotype-dependent and modified by inoculation. Genotype AV1 showed consistently positive responses, AV2 moderate responses particularly under Mn stress, and AV3 limited effects. Metal accumulation patterns suggested genotype-dependent differences, with AV2 showing higher accumulation in roots and lower Mn concentrations, while AV1 and AV3 showed higher Pb concentration in older leaves under inoculated conditions. Overall, the results suggest that plant responses in *A. vulneraria* may depend on specific genotype–rhizobia–metal combinations, but these findings are based on a limited number of genotypes and a single inoculum and should be interpreted cautiously with respect to phytoremediation applications.

Keywords: heavy metals; metal accumulation; nitrogen-fixing bacteria; photochemical performance; phytoremediation



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1. Introduction

Soil contamination with heavy metals such as lead (Pb) and manganese (Mn) represents a major environmental constraint for plant growth and ecosystem functioning. Pb, a non-essential element, is highly toxic to plants, disrupting membrane integrity, cell division, and photosynthetic processes, thereby causing growth inhibition and premature senescence [1–3]. In contrast, Mn is an essential micronutrient involved in enzymatic activity and photosystem II function, but becomes toxic at elevated concentrations, leading to oxidative stress and disturbance of redox balance [4–7]. Due to their contrasting physiological roles, Pb and Mn induce fundamentally different toxicity mechanisms, neces-

situating distinct plant tolerance and detoxification strategies, particularly in contaminated environments where both metals may co-occur.

The majority of legume species (Fabaceae) engage in a specialized form of symbiosis with nitrogen-fixing bacteria, resulting in the formation of root nodules that facilitate the supply of reduced nitrogen compounds to the plant [7]. These highly diverse soil bacteria, collectively referred to as rhizobia, initiate coordinated developmental changes in host roots through an intricate mutual signal exchange system [8]. Rhizobial symbiosis holds immense ecological and agricultural significance, enabling wild legume species to thrive in nitrogen-deficient habitats. However, legume cultivation in cropping systems offers the potential to enhance soil fertility without the excessive application of nitrogen fertilizers [9,10].

Rhizobial symbiosis has also attracted attention in the remediation of degraded or polluted environments [11–13], where it can improve plant tolerance, promote growth, and decrease contaminant mobility [14,15]. The high ecological and functional diversity of wild legume species, on the one hand, and the taxonomic diversity of rhizobial bacteria, on the other, present a wide range of opportunities for the identification of novel model systems for scientific research and subsequent potential practical applications.

Previous studies have demonstrated that *Anthyllis vulneraria* L. encompasses metallicolous genotypes exhibiting remarkable tolerance to Zn, Pb, Cd, and other metals, rendering it an ideal system for investigating plant adaptation to extreme soil conditions. Research on soils contaminated with Zn–Pb reveals that *A. vulneraria* is frequently associated with metal-tolerant *Mesorhizobium* spp., particularly *Mesorhizobium metallidurans* [16,17]. These rhizobia dominate in mine soils and constitute the core of the *A. vulneraria* microsymbiont community, facilitating nitrogen fixation under extreme metal conditions. To date, more attention has been directed specifically towards elucidating the metal tolerance properties of the bacterial partner, including transcriptional responses to the presence of heavy metals [18–21].

A. vulneraria populations can successfully develop on highly contaminated mine tailings and exhibit genotype-specific differences in growth, morphology, and metal accumulation patterns under metal stress [16]. This genotype variability provides a natural experimental framework for analyzing the contributions of both symbiotic partners to tolerance mechanisms. As rhizobial interactions influence plant physiological responses by regulating nutrient acquisition, stress signaling, and root architecture [22–25], understanding the interaction between plant genotype and rhizobacterial species is crucial for identifying plant–microbe combinations that optimize tolerance and metal immobilization.

An ecologically isolated population of *A. vulneraria*, specifically distributed on coastal dunes of the Eastern Baltics, is considered to represent *A. vulneraria* subsp. *maritima* (Hagen) Corb. [26] or even a separate species, *Anthyllis maritima* Schweigg. [27]. Recently, it was established that *A. vulneraria* plants from coastal sand dunes exhibit relatively high tolerance to several heavy metals (Cd, Cu, Zn) in a non-symbiotic state [28]. However, the plants were moderately susceptible to Pb and Mn. Importantly, phytoremediation potential for Cd, Pb, Mn, and Zn was also characterized as relatively high. Given the ecological importance of *A. vulneraria* and the role of rhizobacterial symbiosis in mediating heavy metal tolerance, a targeted comparison of multiple genotypes inoculated with a single bacterial strain under Pb and Mn stress may provide a useful experimental approach to disentangle plant–microbe–metal interactions.

The objective of the present study was to compare the responses of three distinct *A. vulneraria* genotypes to Pb and Mn stress, with a particular emphasis on growth, physiological characteristics, and metal accumulation patterns. It was hypothesized that responses of contrasting *A. vulneraria* genotypes to Pb and Mn are influenced by inoculation

with a specific rhizobial strain, resulting in genotype- and metal-specific differences in plant performance.

2. Results

2.1. Morphological Responses

In this experiment, heavy metal treatment of *Anthyllis vulneraria* commenced only three weeks after inoculation with the bacterial suspension, facilitating the development of symbiosis in the inoculated plants. Following heavy metal treatment, visually discernible variations in morphological characteristics were observed, contingent upon genotype, rhizobia inoculation, and treatment with Pb and Mn (Figure S1). Generally, growth inhibition and accelerated leaf senescence were observed under the influence of Pb, whereas under the influence of Mn, growth reduction and/or leaf curling due to asymmetric cell elongation were evident.

Nodule formation on the roots of *A. vulneraria* exhibited variability among genotypes and metal treatments (Table 1). Under control conditions, genotypes AV1 and AV3 demonstrated consistently high nodulation (score 3), while in AV2, despite high nodulation on the majority of plants, only a limited number of nodules formed on the roots of one individual plant. Pb exposure significantly reduced nodule presence in AV1, whereas AV2 and AV3 maintained high nodulation comparable to the control plants. However, one individual plant of AV2 treated with Pb failed to form nodules. Mn treatment similarly reduced nodulation in AV1 but had no discernible negative impact on AV2 or AV3, which exhibited uniformly high nodule scores across replicates. No nodules were observed in plants not inoculated with rhizobia. These AV2 plants, which did not establish an effective bacterial symbiosis, were visually distinct due to the reduced leaf chlorophyll content (Figure S2). Similarly, AV1 plants, which did not develop effective symbiosis under the influence of Pb, were visually noticeable. However, all plants were included in the analysis irrespective of their nodulation status. The presence of individual plants without effective nodulation within inoculated treatments likely contributed to variability in plant responses and should be considered a limitation of the study.

Table 1. Relative nodulation score for roots of individual plants of *Anthyllis vulneraria* inoculated with rhizobia by treatments.

Genotype	Control		Pb		Mn	
	By Replicates	Mean $\pm$ SE	By Replicates	Mean $\pm$ SE	By Replicates	Mean $\pm$ SE
AV1	3-3-3-3-3	3.0 $\pm$ 0.0	0-2-2-1-3	1.6 $\pm$ 0.5	0-2-2-1-1	1.2 $\pm$ 0.4
AV2	1-3-3-3-3	2.6 $\pm$ 0.4	3-3-3-3-0	3.0 $\pm$ 0.0	3-3-3-3-3	3.0 $\pm$ 0.0
AV3	3-3-3-3-3	3.0 $\pm$ 0.0	3-3-3-3-3	3.0 $\pm$ 0.0	3-3-3-3-3	3.0 $\pm$ 0.0

0, no nodules; 1, a few nodules at only one point; 2, small groups of nodules at several points on roots; 3, a large number of nodules throughout the root length. No nodules were found on plants not inoculated with rhizobia.

Figure S3 summarizes the differences in morphological parameters between treatment combinations for different genotypes. Table S1 provides the statistical evaluation of these differences. To further elucidate the relative effects of rhizobia inoculation and heavy metal treatment on plant morphology, separate tables are presented.

For all *A. vulneraria* genotypes, the number of leaves was significantly influenced by rhizobia and heavy metals, although the interaction effect was statistically significant only for AV1 (Table S1). Dry biomass of leaves was significantly affected by rhizobia for all genotypes, but the impact of heavy metals was significant only for AV1 and AV2. Notably, for AV2, there was a statistically significant interaction between the two factors. In terms of

root biomass, the effects of both rhizobia and heavy metals were statistically significant for all genotypes. The interaction effect on root biomass was significant for AV1 and AV2.

Rhizobial inoculation had a notable impact on plant morphology in *A. vulneraria*, and these responses strongly depended on a genotype and metal treatment (Table 2). Under control conditions, rhizobial inoculation resulted in an increase in the number of leaves across all three genotypes, with comparable relative increases observed in AV1 (+23%), AV2 (+25%), and AV3 (+24%). Leaf dry mass was also significantly enhanced in AV1 (+24%) and AV3 (+19%), while AV2 did not demonstrate a significant response. Root dry mass exhibited the most pronounced response in AV1, where inoculation resulted in a 57% increase, whereas no significant changes were detected in AV2 and AV3 under control conditions.

Table 2. Relative effect of rhizobial inoculation on morphological parameters of different genotypes of control and heavy metal-treated *Anthyllis vulneraria* plants.

Parameter	Genotype	Control	Pb	Mn
Number of leaves	AV1	23	34	56
	AV2	25	55	0
	AV3	24	0	25
Leaf dry mass	AV1	24	0	75
	AV2	0	0	61
	AV3	19	55	21
Root dry mass	AV1	57	57	115
	AV2	0	24	106
	AV3	0	0	0

Data show % increase by rhizobial inoculation over respective non-inoculated plants. Only statistically significant effects ($p < 0.05$) are shown.

Under Pb treatment, rhizobial inoculation significantly increased leaf number in AV1 (+34%) and AV2 (+55%), but not in AV3 (Table 2). Leaf dry mass was positively affected only in AV3 (+55%). Root dry mass increased in AV1 (+57%) and AV2 (+24%) following inoculation, whereas AV3 showed no significant response. Under Mn stress, rhizobial inoculation had pronounced positive effects on plant growth. Leaf number increased significantly in AV1 (+56%) and AV3 (+25%), while no effect was observed in AV2. Leaf dry mass increased in all genotypes, with particularly strong stimulation in AV1 (+75%) and AV2 (+61%). Root dry mass showed the largest inoculation-induced increases under Mn treatment, especially in AV1 (+115%) and AV2 (+106%), whereas AV3 again showed no significant inoculation response.

Exposure to lead (Pb) and manganese (Mn) generally resulted in adverse effects on morphological traits of *A. vulneraria* plants. However, the magnitude of these effects and their modulation by rhizobial inoculation were genotype-specific (Table 3). Pb treatment had minimal impact on leaf number in non-inoculated plants across all genotypes. Notably, in inoculated AV1 plants, Pb induced a significant reduction in leaf number (−27%). Leaf dry mass was significantly reduced by Pb in AV1 and AV2 regardless of inoculation status (reductions of 10–38%). In contrast, in AV3, Pb reduced leaf dry mass only in non-inoculated plants (−22%), while inoculated plants exhibited no significant changes. Root dry mass was strongly reduced by Pb in AV1 (−40%) and AV3 (−25%), with comparable effects in inoculated and non-inoculated plants. Conversely, AV2 demonstrated no significant Pb-induced alterations.

Table 3. Relative effect of heavy metals on morphological parameters of different genotypes of control and rhizobia-inoculated *Anthyllis vulneraria* plants.

Parameter	Genotype	Pb		Mn	
		–R	+R	–R	+R
Number of leaves	AV1	0	–27	–37	0
	AV2	0	0	+25	0
	AV3	0	0	0	0
Leaf dry mass	AV1	–31	–38	–67	–53
	AV2	–12	–10	–38	0
	AV3	–22	0	0	0
Root dry mass	AV1	–40	–40	–84	–78
	AV2	0	0	–58	0
	AV3	–25	–23	0	–56

Data show % changes by heavy metal treatment over respective control plants. Only statistically significant effects ($p < 0.05$) are shown. Blue color indicates that the effect of heavy metal was similar for control and rhizobia-inoculated plants. Red color indicates that the effect of heavy metal was more negative in rhizobia-inoculated plants. Green color indicates that the effect of heavy metal was less negative in rhizobia-inoculated plants.

Table 3 illustrates the more pronounced and variable effects of Mn treatment. In AV1, Mn treatment resulted in a 37% reduction in leaf number and a 67% reduction in leaf dry mass in non-inoculated plants. These adverse effects were partially or fully mitigated by rhizobial inoculation. Conversely, in AV2, Mn treatment led to a 25% increase in leaf number in non-inoculated plants, while inoculated plants exhibited no significant change. Notably, leaf and root dry mass were significantly reduced in non-inoculated AV2 plants under Mn treatment, whereas these reductions were absent in inoculated plants, suggesting a protective effect of rhizobia. In contrast, AV3 did not demonstrate significant Mn-induced alterations in leaf traits. However, Mn treatment resulted in a substantial reduction in root dry mass in inoculated plants (–56%), while non-inoculated plants remained unaffected.

2.2. Physiological Responses

Separate evaluations of water content changes were conducted in various parts of *A. vulneraria* plants (Figure 1). A two-way ANOVA revealed that water content was differentially influenced by rhizobial inoculation and heavy metal treatment in a genotype- and organ-specific manner (see Table S1 for F and p values). In leaves, heavy metals exerted a strong and consistent effect on water content across genotypes, particularly in old and new leaves. Conversely, rhizobial symbiosis significantly modified water content mainly in middle and young leaves, with the most pronounced effects observed in genotypes AV2 and AV3. Significant rhizobia $\times$ metal interactions were primarily detected in leaf tissues, suggesting that symbiotic effects on plant water content were contingent upon metal exposure and genotype. In contrast, root water content was predominantly influenced by heavy metals only in genotype AV1, while rhizobial effects and interactions were generally weak or non-significant in roots across genotypes.

Fluorescence of chlorophyll *a* in the middle leaves of *A. vulneraria* plants was measured twice during the post-treatment period, at weeks 6 and 8. The treatment effects of Performance Index Total (PIT) are depicted in Figure 2, and their statistical evaluation is summarized in Table S1. Rhizobial inoculation and heavy metal exposure induced significant genotype-, metal-, and time-dependent variations in PIT in *A. vulneraria*.

In genotype AV1, the PIT was relatively high in control plants at week 6 and was significantly reduced by both Pb and Mn treatments in non-inoculated plants. Rhizobial inoculation partially mitigated the negative effects of heavy metals at week 6, resulting in higher PIT values compared to non-inoculated plants under both Pb and Mn treatments.

By week 8, inoculated AV1 plants maintained high PIT under control and Pb conditions, whereas Mn treatment caused a pronounced decline, especially in non-inoculated plants.

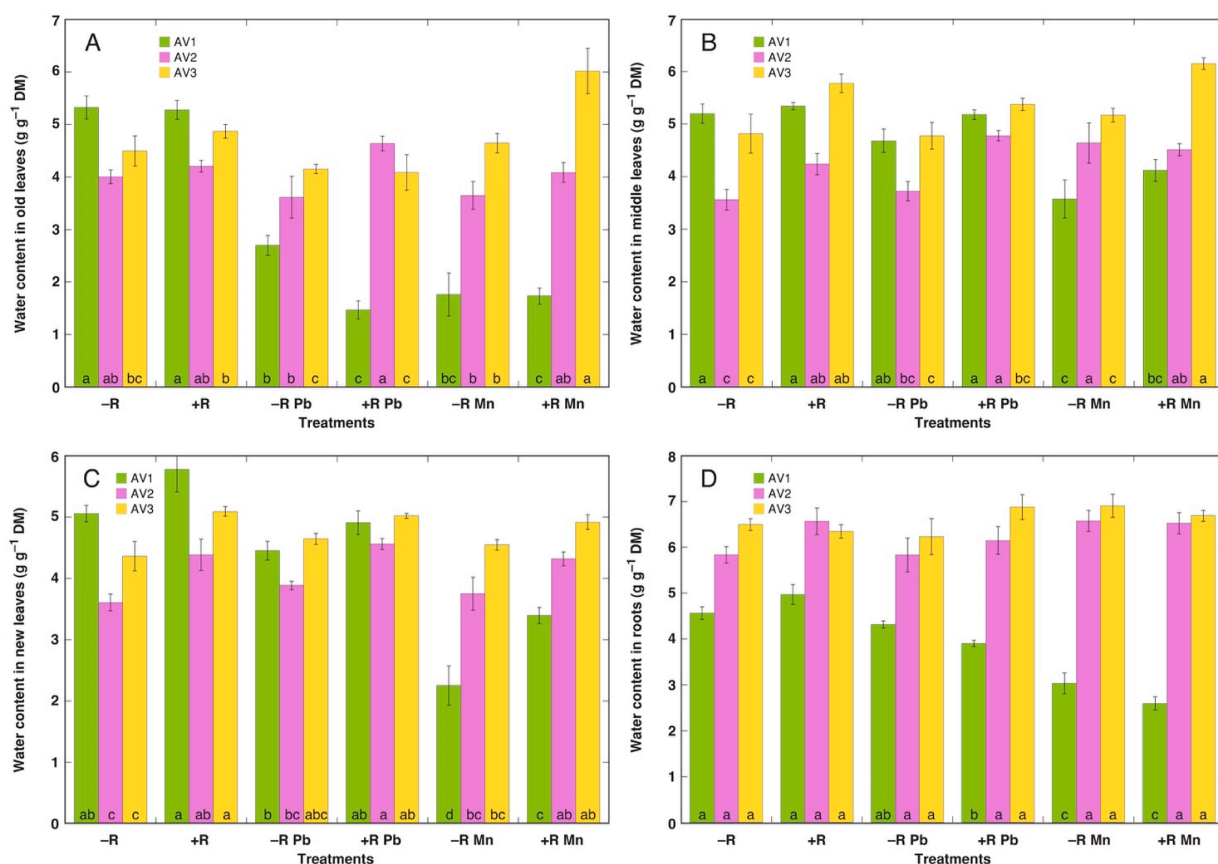


Figure 1. Effect of rhizobial inoculation and heavy metal treatment on water content in different parts of *Anthyllis vulneraria* plants from different genotypes (AV1, AV2, AV3): old leaves (A), middle leaves (B), new leaves (C), roots (D). Data are means $\pm$ SE from five replicates. Different letters indicate statistically significant differences ($p < 0.05$) for each genotype according to the Tukey HSD test.

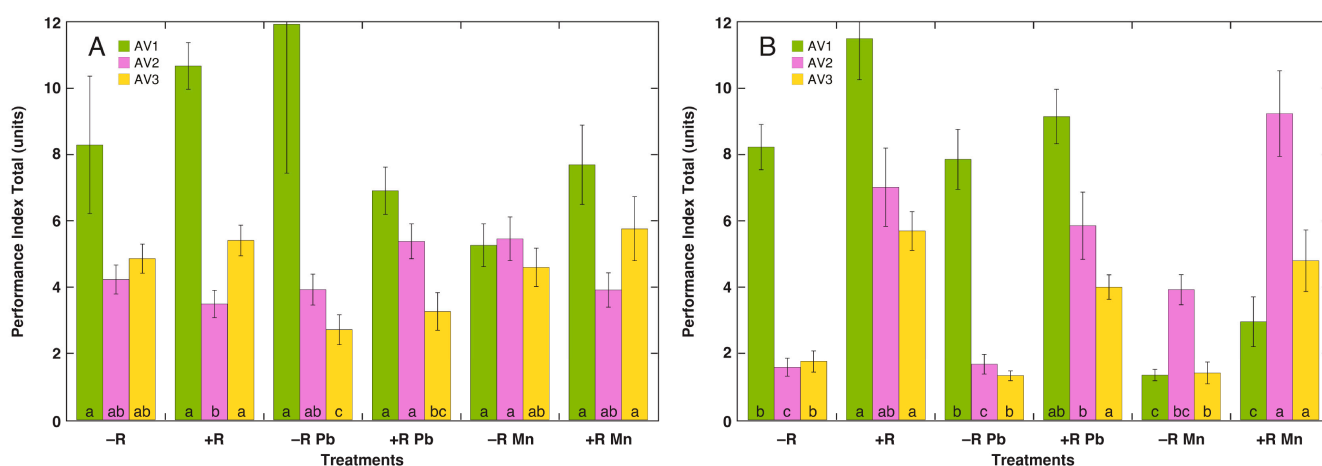


Figure 2. Effect of rhizobial inoculation and heavy metal treatment on chlorophyll *a* fluorescence parameter Performance Index Total in leaves of *Anthyllis vulneraria* plants from different genotypes (AV1, AV2, AV3) at week 6 (A) and week 8 (B). Data are means $\pm$ SE from five replicates with three independent measurements each. Different letters indicate statistically significant differences ($p < 0.05$) for each genotype according to the Tukey HSD test.

In genotype AV2, PIT values were generally lower at week 6 compared to AV1 and exhibited a distinct response pattern. In non-inoculated plants, Mn treatment resulted in higher PIT than control and Pb, whereas rhizobial inoculation at week 6 enhanced PIT primarily under Pb treatment. By week 8, rhizobial inoculation led to a significant increase in PIT across all treatments, with the most pronounced stimulatory effect observed under Mn stress.

In genotype AV3, non-inoculated plants exhibited a moderate PIT at week 6 under control conditions, which was significantly reduced by Pb treatment and to a lesser extent by Mn treatment. Rhizobial inoculation did not enhance PIT at week 6 under Pb treatment but increased values under Mn treatment. By week 8, PIT declined sharply in all non-inoculated treatments, whereas inoculated plants showed substantially higher PIT, particularly under Mn stress.

2.3. Metal Accumulation

Pb accumulation exhibited pronounced organ- and genotype-specific variation in *A. vulneraria* (Table 4). Across all genotypes, Pb concentrations were generally higher in leaves than in roots, with old leaves serving as the primary sink for Pb accumulation. Among non-inoculated plants (−R), genotype AV2 demonstrated the highest Pb accumulation in roots (96.5 mg kg^{-1} dry matter), while AV1 and AV3 exhibited significantly lower root Pb levels. Conversely, AV1 and AV3 accumulated relatively higher Pb concentrations in old leaves, suggesting differences in internal translocation and sequestration strategies among genotypes. New and middle leaves generally contained lower Pb concentrations compared to old leaves, indicating preferential accumulation in older tissues.

Table 4. Effect of rhizobial inoculation on Pb accumulation (mg kg^{-1} DM) in different parts of *Anthyllis vulneraria* plants.

Genotype	Rhizobia	Roots	Old Leaves	Middle Leaves	New Leaves
AV1	−R	$39.1 \pm 4.3 \text{ cd}$	$80.7 \pm 3.3 \text{ b}$	$55.2 \pm 6.3 \text{ c}$	$28.0 \pm 3.9 \text{ de}$
	+R	$22.1 \pm 1.6 \text{ e}$	$139.5 \pm 3.5 \text{ a}$	$18.8 \pm 2.3 \text{ e}$	$18.8 \pm 0.1 \text{ e}$
AV2	−R	$96.5 \pm 3.1 \text{ a}$	$66.8 \pm 4.8 \text{ b}$	$19.5 \pm 2.8 \text{ d}$	$19.9 \pm 3.4 \text{ d}$
	+R	$27.3 \pm 0.1 \text{ cd}$	$32.4 \pm 1.9 \text{ c}$	$19.9 \pm 0.5 \text{ d}$	$31.2 \pm 1.5 \text{ c}$
AV3	−R	$46.6 \pm 4.3 \text{ c}$	$63.0 \pm 7.9 \text{ b}$	$20.2 \pm 0.7 \text{ e}$	$19.6 \pm 1.3 \text{ e}$
	+R	$42.8 \pm 1.1 \text{ c}$	$283.2 \pm 46.0 \text{ a}$	$30.8 \pm 3.9 \text{ d}$	$20.1 \pm 2.3 \text{ de}$

Data are means $\pm$ SE from three replicates. For each genotype, different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). Statistically significant increases in metal accumulation due to inoculation are shown in red, while decreases are shown in blue.

Mn accumulation also exhibited significant variation among plant parts and genotypes, but it followed distinct patterns from Pb (Table 5). In AV1 and AV2, Mn concentrations were highest in leaves, particularly in old, middle, and new leaves, while roots accumulated lower Mn levels. Conversely, AV3 displayed a remarkably different pattern, with roots containing the highest Mn concentrations, whereas leaf Mn levels were comparatively low. Under non-inoculated conditions, AV2 demonstrated the highest overall Mn accumulation in leaves (up to 6.76 g kg^{-1} dry matter in old leaves), followed by AV1. AV3 consistently exhibited the lowest Mn concentrations in leaves but relatively high values in roots, indicating a genotype-specific restriction of Mn translocation to aboveground organs.

Rhizobial inoculation exerted pronounced and genotype-specific effects on lead (Pb) accumulation, with responses varying not only among genotypes but also among plant parts (Table 4). In AV1, inoculation resulted in a significant decrease in root Pb concentration, accompanied by a substantial increase in Pb accumulation in mature leaves. Concurrently, Pb levels in intermediate leaves were notably reduced. In AV2, rhizobial inoculation led to

a general reduction in Pb accumulation in roots and mature leaves, while Pb concentration in new leaves increased significantly. In AV3, inoculation had a relatively minimal impact on root Pb concentrations but induced a dramatic increase in Pb accumulation in mature leaves, along with a moderate increase in intermediate leaves.

Table 5. Effect of rhizobial inoculation on Mn accumulation (g kg^{-1} DM) in different parts of *Anthyllis vulneraria* plants.

Genotype	Rhizobia	Roots	Old Leaves	Middle Leaves	New Leaves
AV1	−R	2.37 ± 0.11 c	4.88 ± 0.01 a	5.03 ± 0.17 a	5.69 ± 0.50 a
	+R	1.55 ± 0.13 d	5.03 ± 0.10 a	3.95 ± 0.15 b	3.66 ± 0.10 b
AV2	−R	4.58 ± 0.25 bc	6.76 ± 0.44 a	6.24 ± 0.25 a	5.64 ± 0.01 ab
	+R	2.24 ± 0.07 e	4.10 ± 0.39 cd	3.15 ± 0.21 d	3.76 ± 0.30 cd
AV3	−R	4.30 ± 0.01 a	1.79 ± 0.14 d	2.33 ± 0.09 bc	2.07 ± 0.13 cd
	+R	4.58 ± 0.54 a	2.59 ± 0.04 b	2.31 ± 0.02 bc	2.12 ± 0.16 cd

Data are means ± SE from three replicates. For each genotype, different letters indicate statistically significant differences according to the Tukey HSD test ($p < 0.05$). Statistically significant increases in metal accumulation due to inoculation are shown in red, while decreases are shown in blue.

In contrast to Pb, rhizobial inoculation had a predominantly negative effect on Mn accumulation in most genotypes and plant parts (Table 5). In AV1, inoculation significantly reduced Mn concentrations in roots, middle leaves, and new leaves, while Mn levels in old leaves remained largely unaffected. AV2 showed the most consistent response, with rhizobial inoculation causing a significant decrease in Mn accumulation in all examined plant parts, including roots and all leaf categories. In AV3, the effect of inoculation on Mn was comparatively weak. Root and younger leaf Mn concentrations remained unchanged, while a modest but significant increase was observed in old leaves.

3. Discussion

3.1. Integrated Conceptual Model

Overall, this study suggests that rhizobial inoculation modulated metal handling in a metal-specific and host genotype-dependent manner in *A. vulneraria* (Figure 3). This modulation was associated with altered Pb redistribution and reduced Mn accumulation. Specifically, rhizobial inoculation reduced Pb retention in roots while promoting Pb sequestration in leaves, particularly old leaves, with variable consequences for plant fitness. Conversely, rhizobia suppressed Mn accumulation in roots and leaves, coinciding with improved growth. These differential responses among plant genotypes illustrate trade-offs between phytoextraction efficiency and growth stability, and may influence suitability for phytoextraction versus phytostabilization strategies. These contrasting mechanisms may help to explain why rhizobia can simultaneously promote metal tolerance, enhance or reduce remediation potential, and impose fitness costs depending on host genotype and metal identity. Such insights underscore the importance of tailored plant–microbe pairings for metal-contaminated environments, where remediation goals must be balanced against sustainable plant growth.

3.2. Genotype-Dependent Modulation of Metal Stress by Rhizobial Symbiosis

The present study clearly suggests that the tolerance of *A. vulneraria* to Pb and Mn stress arises from a genotype-specific interaction between host plants and rhizobial symbionts (Figure 3), rather than from the properties of either partner alone [16,19]. Such genotype-dependent outcomes align with the high specificity that characterizes legume–rhizobia symbioses, where compatibility determines not only nodulation efficiency but also downstream physiological benefits under stress [29,30].

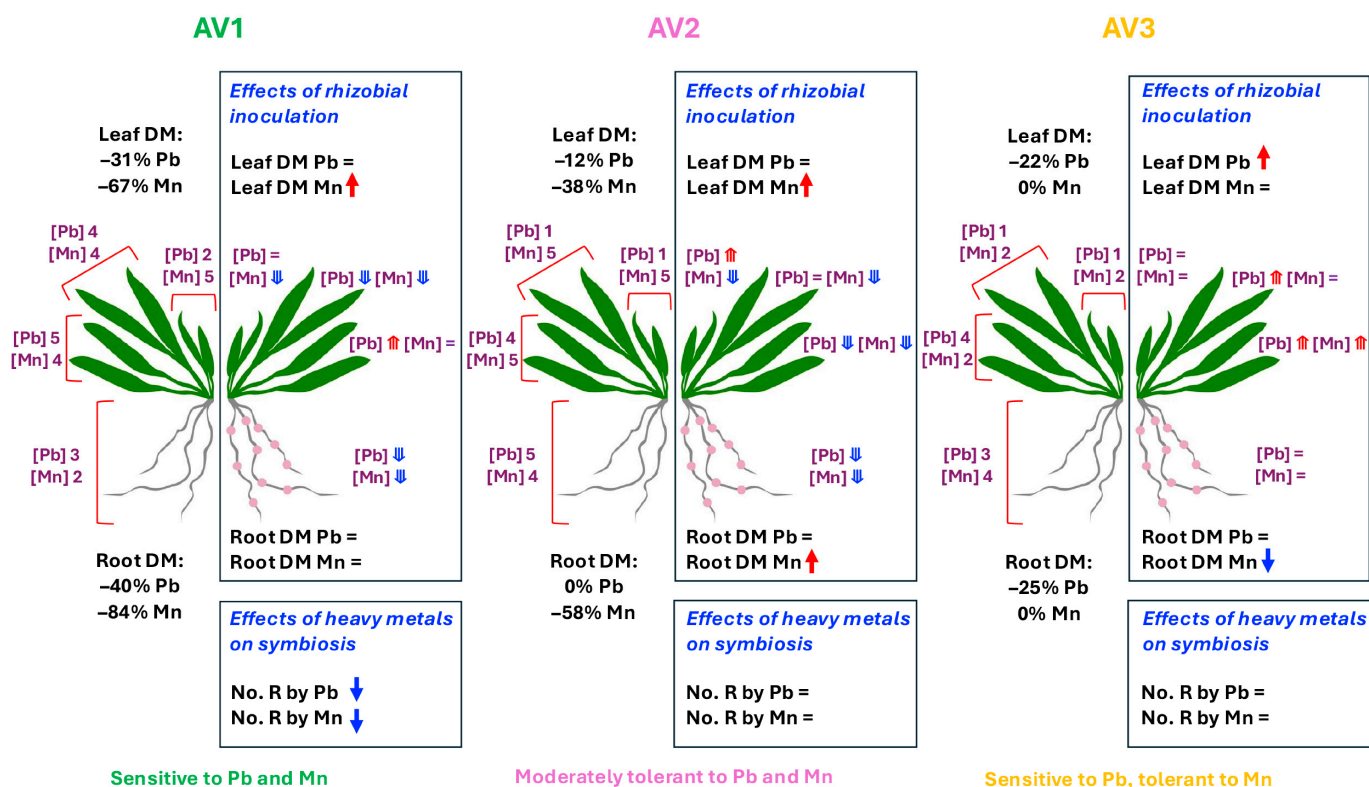


Figure 3. Summary of growth effects of heavy metals and rhizobial inoculation, as well as metal accumulation patterns of *Anthyllis vulneraria* plants of different genotypes. Metal concentration (in square brackets) in different parts of control plants is indicated as a relative rank from 1 to 5 and as significantly increased or decreased due to rhizobial inoculation by corresponding arrows. Red brackets indicate groups of new, middle, and old leaves in the direction from the center to the edge, as well as roots. AV1, *Anthyllis vulneraria* subsp. *maritima*; AV2, *Anthyllis vulneraria*; AV3, *Anthyllis vulneraria* var. *coccinea*.

Rhizobial symbiosis is recognized for its ability to enhance plant performance in nutrient-limited and environmentally stressful conditions by improving nitrogen acquisition and regulating stress-related signaling pathways [31–33]. In the present study, this host genotype-dependent beneficial role became evident primarily under metal exposure, supporting earlier observations that the ecological value of rhizobia extends beyond nitrogen fixation into tolerance mechanisms relevant for degraded or contaminated habitats [14,16,19].

Among the studied genotypes of *A. vulneraria*, AV1 exhibited the most pronounced and consistent positive responses to rhizobial inoculation across growth, physiological performance, and biomass allocation. This suggests a high degree of functional compatibility between AV1 and the inoculated rhizobacterial strain, enabling effective symbiosis even under Pb and Mn stress. Such outcomes align with reports that wild legumes from stressful habitats exhibit strong genotype-specific variation in the ability to benefit from microbial partners [14,15]. It is also important to emphasize that the symbiotic bacteria used in this experiment were isolated from the nodules of *A. vulneraria* subsp. *maritima* plants, while AV1 plants were cultivated from seeds obtained from a different *A. vulneraria* subsp. *maritima* population. There is a high probability that plants of the same genotype in different soils form symbioses with taxonomically different rhizobia, which can also affect compatibility and the effectiveness of the symbiosis [34].

3.3. Heavy Metal Effects on Growth and Nodulation: Limits and Benefits of Symbiosis

Pb and Mn imposed distinct stress signatures on plant morphology, reflecting their contrasting modes of toxicity (Table 3). The Pb-induced growth inhibition and leaf senescence observed in this study are consistent with its known disruption of cell division, membrane stability, and photosynthetic metabolism [1–3]. In contrast, Mn toxicity manifested primarily through growth reduction and leaf deformation, in line with its interference with photosystem II and redox homeostasis [4–6].

Successful nodulation under metal stress appears to be associated with improved plant performance. Notably, reduced nodulation in AV1 plants was observed in response to Pb and Mn exposure, manifesting as visible chlorosis and biomass losses in non-inoculated plants. Conversely, AV2 and AV3 exhibited robust symbiosis, maintaining effective nitrogen fixation. This observation corroborates previous findings indicating that metal-tolerant *Mesorhizobium* strains are prevalent in contaminated soils and possess the capacity to sustain nitrogen fixation under extreme conditions [16,17].

These findings indicate that the presence of a metal-resistant rhizobial partner does not automatically translate into host benefit; however, this conclusion is based on a single strain and highlights the importance of strain-specific compatibility in plant–rhizobia–metal interactions. The extent of symbiotic advantage was significantly influenced by host genotype, underscoring that tolerance mechanisms are co-regulated by both partners rather than being unilaterally imposed by the bacterial microsymbiont [12,18].

3.4. Rhizobia-Driven Modulation of Tissue Water Content and Photosynthetic Performance

Rhizobial effects on plant water content were predominantly observed in shoot tissues rather than roots, particularly under metal stress (Figure 1). This pattern may suggest that rhizobial effects on tissue water content are indirect, potentially associated with changes in plant nutritional status or stress responses; however, these mechanisms were not directly tested [9,15,22].

Similar shoot-focused responses have been documented in other legume systems, where rhizobial symbiosis enhanced cytokinin-mediated regulation of leaf growth and delayed stress-induced senescence [24,31]. The strong genotype dependence of these responses observed here further suggests that rhizobia act as modulators rather than universal enhancers of physiological resilience.

Chlorophyll fluorescence analysis provided mechanistic insights into these effects. Rhizobial inoculation enhanced the Performance Index Total (PIT) under Pb and Mn stress, particularly at later stages of exposure, but this effect was not uniform across genotypes. The improved PIT values in inoculated plants were consistent with improved photochemical performance. Previous reports demonstrated that symbiotic microbes can stabilize photosynthetic machinery and antioxidant defenses under metal stress [24,25,35].

3.5. Genotype-Specific Strategies of Pb and Mn Accumulation

Metal accumulation patterns indicated genotype-dependent variation in Pb and Mn distribution, with additional differences observed between plants with and without rhizobial symbiosis (Tables 4 and 5). AV2 exhibited strong lead retention in roots, a pattern consistent with higher accumulation in roots and enhanced tolerance through limitation of shoot exposure [16]. In contrast, AV1 and AV3 preferentially translocated lead to older leaves, consistent with an accumulation strategy in senescing tissues.

Rhizobial inoculation shifted Pb distribution toward aboveground organs in all genotypes, although the magnitude and direction of this effect varied. Similar patterns have been reported previously; however, the underlying mechanisms remain unresolved and

have been hypothesized to involve changes in rhizosphere chemistry, metal solubility, or transport processes mediated by microbial activity [11,14].

In contrast to Pb, Mn accumulation was generally suppressed by rhizobial inoculation, particularly in AV1 and AV2. Reduced Mn accumulation may reflect differences in Mn availability or plant responses, as suggested in other legume systems [11], but underlying mechanisms were not studied. The weak response of AV3 to inoculation indicates that Mn homeostasis in this genotype is largely controlled by intrinsic plant traits rather than by microbial modulation.

3.6. Trade-Offs Between Plant Fitness and Phytoremediation Potential

The observed divergence in growth responses and metal accumulation patterns suggests a possible trade-off between plant fitness and phytoremediation efficacy. Genotypes such as AV2, which exhibit robust root metal retention coupled with effective rhizobia-mediated stress mitigation, may be more suitable for phytostabilization approaches aimed at restricting metal mobility in contaminated soils [13,15]. Conversely, AV1 and AV3, which demonstrate enhanced shoot Pb accumulation under symbiotic conditions, may be more appropriate candidates for phytoextraction strategies, albeit with potential drawbacks to long-term plant performance. These interpretations should be considered preliminary, as the study did not assess total metal uptake, long-term stability, or field performance.

These findings suggest that successful legume-based phytoremediation may depend on careful matching of plant genotype, microbial partner, and target metal; however, this conclusion is based on the specific combinations and experimental conditions tested here and should not be generalized without further validation [11,12]. *A. vulneraria*, characterized by its pronounced intraspecific variability and adaptable symbiotic responses, thus emerges as a valuable model for the development of genotype-informed, sustainable remediation strategies for metal-contaminated environments.

4. Materials and Methods

4.1. Plant and Microbiological Material

Three different genotypes of *Anthyllis vulneraria* were used in the present study. Seeds of *Anthyllis vulneraria* subsp. *maritima* (AV1) were collected on coastal dunes near Oviši, Latvia (57°34'31.9" N, 21°43'16.5" E). Seeds of *Anthyllis vulneraria* (AV2) and *Anthyllis vulneraria* var. *coccinea* (AV3) were purchased from Jelitto Staudensamen GmbH (Schwarmstedt, Germany).

Rhizobia were obtained from wild-grown *A. vulneraria* subsp. *maritima* plants collected in November in the coastal dunes of Jūrmala in Lielupe, Latvia (57°00'16.7" N, 23°55'21.1" E). Bacterial inoculant for plant treatment was obtained according to the methodology described previously [35]. The inoculum was taxonomically identified based on previous characterization [35]. Culture purity was verified prior to use, and inoculum was produced under standard laboratory conditions.

4.2. Plant Propagation and Growth Conditions

The experiment was performed during the winter season in partially controlled conditions of an experimental greenhouse. Propagation of plant material for experiments and plant cultivation were carried out according to the procedures described earlier [28,35]. Briefly, seeds were surface sterilized using diluted household bleach and scarified, before sowing in sterile plastic tissue culture containers with sterilized commercial substrate (Garden Soil, Biolan, Eura, Finland). Containers were incubated in a growth cabinet at 23 °C in low light conditions. After appearance of the first true leaves, individual seedlings were transplanted to 200 mL plastic containers with a mixture (3:2, *v/v*) of Garden Soil and

quartz sand (Saulkalne-S, Saulkalne, Latvia), placed in 48 L plastic boxes and accommodated to greenhouse conditions using gradual ventilation. After two weeks, plants were individually transplanted to 1.2 L containers with the same substrate.

Plants were cultivated in a greenhouse (MortiMaX, Maasdijk, The Netherlands) with supplemented light (photon flux density of photosynthetically active radiation $380 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the plant level), for a 16 h photoperiod, at day/night temperature 23/16 °C, and a relative air humidity of 60 to 70%. Substrate water content was monitored daily with an HH2 moisture meter equipped with WET-2 sensor (Delta-T Devices, Burwell, UK) and maintained at 50 to 60% of volumetric water content using deionized water.

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.

4.3. Inoculation and Treatments

One week after the final transplanting, plants were assigned to six treatment groups, five replicates (individual containers) per treatment (Table 6). After that, the respective plants were inoculated with a bacterial suspension (about 10^9 colony-forming units per mL; estimated by a standard plate count method), applied in six points (1 mL each) per container over the surface of the substrate. Treatment with heavy metals was carried out two separate times, three and four weeks after rhizobial inoculation. Two heavy metals, Pb and Mn, were selected for treatments, based on their pronounced negative effect on *A. vulneraria* plants in similar experimental conditions [28]. The lowest concentration that caused a negative effect on growth was used, 500 mg of the respective metal, Pb or Mn, per L of substrate (Table 6). These concentrations were selected as experimental stress levels and may not directly correspond to natural field conditions. The indicated amount of respective salt was dissolved in deionized water and 0.1 L per container was applied to the substrate surface in each container. Plants were cultivated for four additional weeks after the full treatment with heavy metals. During this period, the morphological characteristics of visually assessed average plants from each treatment were photographed once a week.

Table 6. Treatments used in the present study with *Anthyllis vulneraria* plants.

Treatment	Rhizobial Inoculation	Salt	Total Amount of Salt (g per L of Substrate)	1st Treatment (g per L of Substrate)	2nd Treatment (g per L of Substrate)
Control –R	–	–	–	–	–
Control +R	+	–	–	–	–
Pb –R	–	PbOAc $3\text{H}_2\text{O}$	0.916	0.366	0.550
Pb +R	+	PbOAc $3\text{H}_2\text{O}$	0.916	0.366	0.550
Mn –R	–	MnSO ₄ H_2O	1.500	0.600	0.900
Mn +R	+	MnSO ₄ H_2O	1.500	0.600	0.900

4.4. Measurement of Chlorophyll *a* Fluorescence

The chlorophyll *a* fluorescence was measured in leaves dark-adapted for at least 20 min with a Handy PEA fluorometer (Hansatech Instruments, King's Lynn, UK). Three measurements of fast fluorescence induction per individual plant were performed. Fluorescence data analysis was conducted utilizing PEA Plus software (version 3.11, Hansatech Instruments, King's Lynn, UK). To characterize photochemical performance of photosynthesis, Performance Index Total was used. This complex parameter combines four

function-related parameters (trapping of absorbed exciton, electron transport between the photosystems, reduction in end-electron acceptors, and the status of photosystem I) [36].

4.5. Termination of the Experiment

At harvest, the plant leaves were classified into three age categories based on their position in the shoot, degree of expansion, and visual appearance. Young leaves were defined as recently emerged, upright, located in the center of the shoot, and not fully expanded leaves with light green coloration. Middle leaves were fully expanded, located to the edge of the new leaves, and uniformly dark green. Old leaves were basal leaves, usually horizontally oriented, exhibiting signs of senescence, including yellowing or chlorosis and reduced turgor. Leaves were counted and weighed separately. The roots were separated from the substrate and washed to remove any soil particles. The relative degree of nodule presence was evaluated according to the four point scale (0, no nodules; 1, a few nodules (1–5) at only one point; 2, small groups of nodules (<10) at several points on roots; 3, a large number of nodules (>10) throughout the root length). This measure was not intended as a quantitative assessment of symbiotic efficiency, and more detailed parameters (e.g., nodule number, biomass, or activity) were not evaluated in this study. All plants were included in the analysis irrespective of their nodulation status.

Plant material was weighed separately before and after drying in an oven at 60 °C for 72 h. The tissue water content was estimated as a mass of water in grams per gram of dry mass.

4.6. Analysis of Heavy Metals

Heavy metal concentrations in shoot and root samples were measured in triplicate as described in detail previously using a microwave plasma atomic emission spectrometer (4200 MP-AES, Agilent, Santa Clara, CA, USA) [37]. From each treatment, three individual plants were randomly selected for analysis, which represents a limitation for interpretation of metal accumulation data.

4.7. Data Analysis

The data were analyzed by KaleidaGraph (v. 5.0, Synergy Software, Reading, PA, USA). All measured parameters satisfied the assumptions of normality and homogeneity of variance according to the Shapiro–Wilk and Levene tests. Statistical significance of differences for measured parameters between treatments was evaluated by two-way ANOVA followed by post hoc analysis by Tukey honestly significant difference test. The two factors considered were rhizobial inoculation and treatment with heavy metals. Analyses were performed separately for each *A. vulneraria* genotype. Significant differences were indicated at $p < 0.05$.

5. Conclusions

This study compared responses of three *Anthyllis vulneraria* genotypes to Pb and Mn stress in the presence or absence of rhizobial inoculation with a single strain. Pb and Mn negatively affected plant performance, and responses differed among genotypes and inoculation treatments. AV1 showed relatively strong responses to inoculation, AV2 moderate responses, and AV3 limited effects. Metal accumulation patterns suggested different distribution among plant parts, with AV2 showing higher accumulation in roots and AV1 and AV3 higher Pb concentration in older leaves. Overall, the results indicate that plant responses may depend on specific genotype–rhizobia–metal combinations; however, conclusions are based on a limited number of genotypes and one inoculum and should be interpreted cautiously. This underscores the importance of genotype-informed selection of symbiotic partners for both ecological adaptation and remediation applications. Fu-

ture studies should expand this model by testing multiple rhizobial strains, integrating molecular and ionic approaches to elucidate tolerance mechanisms, and validating genotype-specific responses under long-term and field conditions. Such work will further establish *A. vulneraria* as a valuable system for mechanistic and applied studies of plant–microbe–metal interactions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/stresses6030040/s1>. Figure S1: Morphological changes in typical *Anthyllis vulneraria* plants due to rhizobial inoculation (+R) and heavy metal treatment (Pb, Mn); Figure S2: Overview of all *Anthyllis vulneraria* plants on the final day of the experiment; Figure S3: Effect of rhizobial inoculation and heavy metal treatment on number of leaves (A), dry biomass of leaves (B), and dry biomass of roots (C) of *Anthyllis vulneraria* plants from different genotypes (AV1, AV2, AV3); Table S1: The results of two-way ANOVA of morphological and physiological parameters of different *Anthyllis vulneraria* genotypes as affected by inoculation with rhizobia and treatment with heavy metals (Pb or Mn).

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Abbreviations

The following abbreviations are used in this manuscript:

AV1	<i>Anthyllis vulneraria</i> subsp. <i>maritima</i>
AV2	<i>Anthyllis vulneraria</i>
AV3	<i>Anthyllis vulneraria</i> var. <i>coccinea</i>
PIT	Performance Index Total

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