



Zinc accumulation in *Atriplex lentiformis* is driven by plant genes and the soil microbiome

Priyanka Kushwaha^{a,*}, Alexandria Tran^b, Diego Quintero^b, Miranda Song^b, Qi Yu^{b,1}, Ruth Yu^c, Michael Downes^c, Ronald M. Evans^c, Alicja Babst-Kostecka^a, Julian I. Schroeder^b, Raina M. Maier^a

^a Department of Environmental Science, The University of Arizona, Tucson, AZ 85721, USA

^b School of Biological Sciences, Department of Cell and Developmental Biology & Department of Bioengineering, University of California San Diego, La Jolla, CA 92093, USA

^c The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

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ABSTRACT

Successful phytoremediation of acidic metal-contaminated mine tailings requires amendments to condition tailings properties prior to plant establishment. This conditioning process is complex and includes multiple changes in tailings bio-physico-chemical properties. The objective of this project is to identify relationships between tailings properties, the soil microbiome, and plant stress response genes during growth of *Atriplex lentiformis* in compost-amended (10 %, 15 %, 20 % w/w) mine tailings. Analyses include RNA-Seq for plant root gene expression, 16S rRNA amplicon sequencing for bacterial/archaeal communities, metal concentrations in both tailings and plant organs, and phenotypic measures of plant stress. Zn accumulation in *A. lentiformis* leaves varied with compost levels and was the highest in the intermediate treatment (15 %, TC15). Microbial analysis identified *Alicyclobacillus*, *Hydrothalea*, and *Pseudolabrys* taxa with the highest relative abundance in TC15, and these taxa were strongly associated with Zn accumulation. Furthermore, we identified 190 root genes with significant gene expression changes. These root genes were associated with different pathways including, abscisic acid and auxin signaling, defense responses, ion channels, metal ion binding, oxidative stress, transcription regulation, and transmembrane transport. However, root gene expression changes were not driven by the increasing levels of compost. For example, there were 15 genes that were up-regulated in TC15, whereas 106 genes were down-regulated in TC15. The variables analyzed explained 86 % of the variance in Zn accumulation in *A. lentiformis* leaves. Importantly, Zn accumulation was driven by Zn shoot concentrations, leaf stress symptoms, plant root genes, and microbial taxa. Therefore, our results suggest there are strong plant-microbiome associations that drive Zn accumulation in *A. lentiformis* and different plant gene pathways are involved in alleviating varying levels of metal stress. Future work is needed to gain a mechanistic understanding of these plant-microbiome interactions to optimize phytoremediation strategies as they will govern the success or failure of the revegetation process.

1. Introduction

Legacy mine sites often contain weathered, oxidized, sulfide-rich tailings contaminated with high levels of metal(loid) contaminants as a byproduct of past, less efficient mining operations (Dybowska et al., 2006; Lu and Wang, 2012). Characterized by small particle size, low carbon and nutrient content, poor soil structure, high metal(loid)

concentrations, acidic pH, and an acidophilic lithoautotroph-dominated microbial community, these mine tailings are often resistant to natural plant establishment and growth (Mendez et al., 2007; Valentín-Vargas et al., 2014). Consequently, these tailings are susceptible to wind dispersion leading to human health risks arising from metal(loid)-containing particulates, especially in arid environments (Mendez and Maier, 2008a; Root et al., 2015).

* Corresponding author.

E-mail address: pkushwaha@arizona.edu (P. Kushwaha).

¹ Present address: School of Life Sciences, Central China Normal University, Wuhan, Hubei, China.

Phytostabilization is a remediation technology used to stabilize contaminated tailing materials against wind and water erosion by planting directly into amended tailings (Mendez and Maier, 2008b; Gil-Loaiza et al., 2016, 2018). Plants are specifically selected for their capacity to survive in the tailings and to immobilize metal(loid)s in the root zone rather than accumulating them in the aboveground plant material (Mendez et al., 2007; Solís-Dominguez et al., 2012). Phytostabilization is often supported by addition of soil amendments (Mendez et al., 2007; Conesa et al., 2012; Eissa, 2015), in particular organic amendments support plant growth by increasing the pH, organic carbon levels, overall soil bacterial biomass and diversity (Mendez et al., 2007; Grandlic et al., 2008; Solís-Dominguez et al., 2012; Valentín-Vargas et al., 2014; Gil-Loaiza et al., 2016; Eissa et al., 2022). Previous research has demonstrated that increased pH is a driver for plant success in acidic tailings through significant reductions in the acidophilic lithoautotrophic Fe/S oxidizing community that is responsible for acidification of tailings (Valentín-Vargas et al., 2014, 2018; Honeker et al., 2017, 2019; Hottenstein et al., 2019).

Current understanding of phytostabilization is based primarily on the bio-physico-chemical characteristics of the mine tailings being treated and the amendments that are applied. However, multiple studies have demonstrated that plants have the capacity to respond differently to varying levels and types of stress in mine tailings (e.g., pH, metal content) (Mendez et al., 2007; Santala and Ryser, 2009; Solís-Dominguez et al., 2012). This suggests that critical information is missing on plant response and their influence on the phytostabilization process. Specifically, little is known about the plant-soil-metal-microbiome interactions during the phytostabilization process, especially during the critical phase of early plant establishment.

In the present study, we address this knowledge gap by simultaneously studying both bacterial/archaeal diversity and plant gene expression during phytostabilization of mine tailings. The plant chosen for this study is *Atriplex lentiformis* (Torr.) S. Wats. (commonly known as quailbush; Fig. S1) - a native, halophytic, and drought-tolerant perennial shrub of the southwestern United States and Mexico (USDA, NRCS, n.d.; Jordan et al., 2009; Eissa, 2017) that is known to limit metal uptake of most metals into shoots with the exception of zinc (Zn) (Solís-Dominguez et al., 2012). The objectives of the present study are to: 1) compare microbial diversity and composition in rhizosphere and bulk substrates across three different levels of compost (10 %, 15 %, 20 % w/w) amendment; 2) identify and characterize differentially expressed genes in roots of plants grown in the three levels of compost amendment; and 3) evaluate Zn accumulation in *A. lentiformis* by integrating soil chemistry, plant metrics, root gene expression, and rhizosphere microbiome data. We anticipate that these associations will serve as a precursor for designing future hypothesis-driven experiments that can help tease out direct interactions between plant genes, soil nutrients, metal content, and specific substrate microbes. In addition, by providing a deeper understanding of how stress levels in mine tailings affect the plant-associated microbial communities, plant gene expression, and Zn accumulation, this study has implications for phytostabilization management practices.

2. Material & methods

2.1. Greenhouse experimental setup

A greenhouse experiment was performed to determine the changes in the rhizosphere-influenced microbial community of *A. lentiformis* grown in metalliferous tailings amended with compost. Three levels of compost amendment were applied: 10 %, 15 %, and 20 % (w/w) compost, and corresponded to three tailings-compost mixture treatments: TC10, TC15, and TC20, respectively. A 0 % compost treatment was not set-up as a baseline as *A. lentiformis* does not germinate in highly acidic mine tailings. The source of the mine tailings was the Iron King Mine and Humboldt Smelter Superfund (IKMHSS) site in Dewey-Humboldt, AZ.

The tailings were collected from a depth of 0–30 cm and homogenized prior to adding the plant-based compost (Tank's Green Stuff in Tucson, AZ). Four-liter plastic pots with drainage were filled with the tailings-compost mixtures, with 10 replicates per treatment. Due to the low germination rate of *A. lentiformis*, 20 seeds (Phoenix Desert Nursery, Phoenix, AZ) were sown in each pot at a depth of ~0.5 cm in a circular manner with the intent to have one seedling in each pot. Additionally, a set of unseeded pots filled with the tailings-compost mixtures (TC10, TC15, TC20) and tailings only (TO) were included as controls. The pots were randomized across the replicates and treatments. A screen (65–70 % light absorbing) was used to reduce the evaporation until the seeds had germinated. Approximately 10 days after germination, pots were thinned to one seedling and plants were cultivated for an additional ~9 weeks. Pots were watered three times a day (7 am, 12 pm, and 4 pm) with tap water using an automated drip irrigation (~110 mL per pot every day). Plants were grown under natural light conditions with an average day/night length of 13 h/11 h and an average day/night temperature of 40 °C/17 °C. The experiment was conducted at the University of Arizona (UA) Controlled Environment Agriculture Center's (Tucson, AZ) greenhouse from July to October 2018.

2.2. Sample collection: plants and tailings

Eleven weeks following plant germination, four plants from each treatment were randomly selected for microbiome analysis of the tailings-compost mixture and three plants were selected for root gene expression analysis (Fig. S2). Prior to plant harvest, the phenotypic variability in response to tailings exposure was assessed by scoring four traits in each plant: plant height, number of branches, length of the longest branch, and qualitative score for visible symptoms of tailings toxicity on leaves (n = 12). Each pot was cut open, the plant was carefully excavated, and the shoots were separated from the roots. Substrate attached to the roots was sterily collected into a sterile 50 mL tube to represent the rhizosphere-influenced (hereafter referred to as rhizosphere) samples (n = 12) for microbial analysis (Solís-Dominguez et al., 2012). Similarly, a bulk sample (n = 12) was collected from the pot avoiding the plant roots. Samples for microbial analysis were transported on ice to the lab and stored at –80 °C until DNA extraction. After collecting substrate samples, plant roots were washed with Milli-Q water, dried with paper towel, and flash frozen in liquid nitrogen for RNA extraction and RNA-seq analysis (n = 9). Root samples for RNA analysis were transported on dry ice to the University of California, San Diego (UCSD) lab and stored at –80 °C. The corresponding plant shoot, leaf, and root samples were collected for metal(loid) content analyses. Finally, a bulk sample of the tailings-compost mixture was collected from each treatment and placed in a whirl pack bag for chemical analyses (n = 12).

2.3. Chemical analyses of the tailings and plant samples

The unamended and compost-amended IKMHSS mine tailings were analyzed for pH, organic matter, estimated nitrogen release, organic carbon, available nitrogen (NO₃-N and NH₄-N), available phosphorus, total exchange capacity with % base saturation of cations (Ca, Mg, K, Na, H, and other bases), extractable elements (S, P, Ca, Mg, K, Na, B, Fe, Mn, Cu, Zn, Al), and total arsenic (As), lead (Pb), and zinc (Zn) concentrations. These analyses were performed at the beginning (tailings (n = 1), compost (n = 1), and tailings-compost mixtures (n = 3)) and at the end of the experiment (tailings-compost mixtures; n = 15). Each sample was air-dried and sieved to 2 mm. The pH was measured from a 1:1 mass ratio extract of substrate to water (McLean, 1982). Loss on ignition method was used to estimate the organic matter and estimated release of nitrogen (Schulte and Hopkins, 1996), while the total organic carbon was measured by combustion of acid treated samples (Nelson and Sommers, 1996). The available nitrogen (NO₃-N and NH₄-N) and available phosphorus were measured as per Dahnke and Johnson (1990).

and Bray II method (Bray and Kurtz, 1945), respectively. The total exchange capacity in addition to the percent base saturation of cations was estimated as per Ross (1995). Lastly, Mehlich III method was used to measure extractable S, Ca, Mg, K, Na, B, Fe, Mn, Cu, Zn, Al, and P (Mehlich, 1984). All soil chemistry analyses except total metal concentrations were conducted at the Brookside Laboratories, Inc. (New Bremen, OH).

For total As, Pb, and Zn concentration analysis, 20 g of oven-dried substrate mixture was finely ground (McGee et al., 1999), digested using microwave-assisted US-EPA method 3051, and determined with ICP-MS (ELAN DRC-II, Perkin Elmer, Shelton, CT) analysis. Plant samples were divided into roots, shoots, and leaves, dried at 65 °C for 72 h, microwave digested using EPA Method 3051, and total concentrations of As, Pb, and Zn were determined by ICP-MS. All metal analyses were conducted at the Arizona Laboratory for Emerging Contaminants (UA). The significant differences in soil chemistry and plant metal concentrations were tested using ANOVA and Tukey's HSD posthoc test.

2.4. Microbial community analyses

2.4.1. DNA extraction and amplicon sequencing analyses

DNA was extracted from 0.5 g of substrate using the FastDNA Spin Kit for Soil™ (MP Biomedicals, Solon OH, USA) with modifications as described in Kushwaha et al. (2021) to enhance the DNA yield. Samples were thawed on ice and homogenized prior to the extraction. Low DNA biomass samples from TC10 and TO samples were extracted using the combined extraction protocol (Sengupta et al., 2019). Briefly, three replicate DNA extractions were performed per sample and were combined in equal volumes prior to purifying DNA using DNeasy PowerClean Pro Cleanup kit (Qiagen, Hilden, Germany). Purified DNA was quantified using the Qubit 2.0 Fluorometer and double stranded DNA (dsDNA) high sensitivity assay kit (Invitrogen, Carlsbad, California, USA). A negative control sample (blank) containing only reagents was included in each DNA extraction set. The primers 515F/806R (bacteria/archaea) were used to amplify the V4 region of the 16S rRNA gene (Walters et al., 2016). After purifying PCR products, all the samples were pooled together in equimolar concentrations. The paired-end (read length of 2 × 150-bp) sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA) at the Microbiome Core (UA).

2.4.2. Data processing, quality assessment, and diversity analysis

Bioinformatics analysis and taxonomic assignment for the phylo-types (amplicon sequence variants (ASVs)) were performed using the DADA2 pipeline (Callahan et al., 2016) as described in Kushwaha et al. (2021). After removing poor quality and chimeric ASVs, a total of 1,577,145 sequence reads were retained with an average of 47,792 ± 30,896 per sample. ASVs that did not match to a bacterial/archaeal domain or matched to chloroplast/mitochondria were removed. Lastly, removal of contaminants from the ASV table by comparing samples and blanks, resulted in 3651 ASVs for bacterial/archaeal communities (hereafter referred to as microbial communities). The taxonomy table was normalized using a cumulative-sum scaling approach (Paulson et al., 2013). Raw sequences were deposited to NCBI's Sequence Read Archive under the BioProject accession number: PRJNA839855.

Microbial richness/number of observed ASVs, Shannon diversity index, and community dissimilarity were calculated using the vegan package in R (Oksanen et al., 2008). Bray-Curtis distance was used to calculate community dissimilarity and non-metric multidimensional scaling (NMDS) was used for visualization. The differences in microbial richness and Shannon diversity across the treatments (TC10, TC15, and TC20) and sample type (rhizosphere vs. bulk) were determined using the Kruskal-Wallis test. Permutational multivariate analysis of variance (PERMANOVA) was used to assess the effect of treatment and sample type on the microbial beta-diversity (Anderson, 2001).

2.4.3. Microbial community abundance changes

Microbial taxa with significant changes in abundance across treatments were estimated using the DESeq2 package in R for the rhizosphere samples (Love et al., 2014). The ASVs were agglomerated at the taxonomic level of genus to identify significantly abundant genera and only those genera that had a read count >0 in at least two of the three replicates in each treatment were kept (genera = 130). We applied likelihood ratio test (LRT) in the DESeq2 analysis to compare the three treatments. For LRT, two models per microbial genus were estimated and then the two models were compared using an Analysis of Deviance. Since LR follows a Chi-square distribution pattern, an associated *p*-value was calculated by the test for each genus. The *p*-values were adjusted for false discovery rate (FDR) using Benjamini-Hochberg with a cutoff of 0.05 for identifying significantly abundant taxa (Benjamini and Hochberg, 1995).

2.5. Plant gene expression analysis

2.5.1. RNA extraction and sequencing

Total RNA extraction from 0.2 g *A. lentiformis* plant root samples was carried out using RNeasy Plant Mini kit (Qiagen, MD, USA) with modifications. All consumables used for RNA extraction were RNAase free. Modifications included the following: (i) 600 µL of Buffer RLC was used to disrupt the root sample; (ii) RNA was eluted with 35 µL RNase-free water; and (iii) all the centrifugation steps were carried out at 12,000 rpm for 1 min. RNA quantity and quality were determined using NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Sequencing libraries were prepared from 500 ng of total RNA using the TruSeq Stranded Total RNA sample preparation kit (Illumina, CA, USA) according to the manufacturer's protocol. Samples were ligated to unique adaptors and subjected to PCR amplification. Libraries were then validated using the Agilent 2100 BioAnalyzer, normalized, and pooled for sequencing. RNA-seq libraries prepared from three biological replicates for each experimental treatment were subjected to single-ended sequencing on the Illumina HiSeq 2500 using barcoded multiplexing and a 100 bp read length. Image analysis and base calling were performed using Illumina CASAVA-1.8.2. The library prep and sequencing were performed at the Salk Institute for Biological Studies (CA, USA).

2.5.2. RNA-seq quality control, read mapping, filtering, and annotation

Raw sequences (single-reads) were checked for their quality using FastQC (Andrews, 2010). The RNA-seq analysis pipeline is represented as a flowchart in Fig. S3. A *de novo* transcriptome was built using 24 samples that were sequenced together but the RNA-seq analysis pipeline in this study focused on nine root samples, collected from the three compost treatments. The total number of reads for the nine samples were 115,987,934 (112,966,723) with 12,887,548 ± 2,052,508 (12,551,858 ± 1,915,057) average number of reads per sample (counts after removal of Illumina adapters using TrimGalore (<https://github.com/FelixKrueger/TrimGalore>) are given in brackets). Raw (Fig. S4A) as well as trimmed-reads (Fig. S4B) had an average quality of >30 phred score. Post-removal of adapters, reads were corrected by removing erroneous K-mers using Rcorrector (Song and Florea, 2015) and assembled into a *de novo* transcriptome using Trinity version 2.12.0 (Grabherr et al., 2011), that resulted in 153,416 transcripts in total for nine samples. The transcripts were quantified for each sample by pseudo-alignment to the assembled reference transcriptome using the computational tool Kallisto (Bray et al., 2016). Since *A. lentiformis* does not have an annotated transcriptome, BLASTX was used to identify orthologous genes against the UniPROTKB/SWISS-PROT database (The UniProt Consortium, n.d.). Out of the 52,169 *A. lentiformis* transcripts that had a match in the SWISS-PROT database, the maximum matches (62 %) were against the *Arabidopsis thaliana* sequences. Thus, the transcripts with sequence homology to *A. thaliana* genome were only used for downstream analysis. A total of 32,246 distinct transcripts matched to 7771 unique *A. thaliana*

genes in the SWISS-PROT database. Genes named in this study will be referenced as the closest *A. thaliana* ortholog.

2.5.3. Gene expression analysis and functional annotation

The output from Kallisto was imported into DESeq2 using the txim-port package and a normalized count table was generated (Love et al., 2022). The raw counts of the different isoforms of the same gene annotation were summed and rounded off to the nearest integer. Further, the raw counts of the transcripts were summed at the gene level. Genes were filtered and only those that had a count in at least two of the three replicates in each treatment were kept (genes = 4986). Genes with significant changes in gene expression across the three compost treatments were estimated using DESeq2 (Love et al., 2014) as described above for identifying significantly abundant microbial taxa. Principal component analysis (PCA) plot (on scaled normalized count) was generated to visualize the distribution of the significantly different genes and to examine sample clusters within the treatment.

Functional annotation was obtained by uploading the list of differentially expressed genes to the web-based UniProt database (<https://www.uniprot.org/uploadlists/>) and selecting to view the gene ontology for the differentially expressed genes. To further characterize the functions of the genes, the gene IDs were searched against the *A. thaliana* ePlant database (<https://bar.utoronto.ca/epland/>). The raw sequencing and processed data for the *A. lentiformis* transcriptome have been submitted to NCBI under the BioProject ID: PRJNA859667 and Gene Expression Omnibus (GEO) ID: GSE208352, respectively.

2.6. Integrated analysis of soil chemistry, plant metrics, root gene expression, and changes in the substrate microbiome

Partial Least Square (PLS) regression models were used to investigate the contribution of both abiotic and biotic factors and plant metrics to the variation of Zn in *A. lentiformis* leaves as described in Kushwaha et al. (2022). The analysis was run on a response variable, i.e., Zn leaf concentration and a matrix of 268 explanatory variables. The predictors included 29 soil chemistry variables, 12 plant metrics, including plant root, shoot, and leaf metal(loid)s concentration, as well as 226 biotic variables. The biotic variables included: 1) microbial diversity, e.g., richness, Shannon diversity index, and NMDS axes scores; 2) relative abundance of significantly different rhizosphere microbial genera across treatments; and 3) normalized gene count of significantly different genes across treatments. As there were three replicates instead of four per treatment for the gene count, an average of the three replicates was used as the fourth value for the PLS analysis. PLS was performed using XLSTAT.

3. Results

3.1. Effect of compost amendment on the chemistry and metal content of tailings

Unamended IKMHSS tailings had an initial pH of 3.1 while the compost had a pH of 7.4 (Table S1). Immediately following compost amendment, the tailings pH increased to 3.5, 4.3, and 5.2 in the TC10, TC15, and TC20 treatments, respectively. The pH continued to slightly increase during the experiment to 4.0 ± 0.1 (TC10), 5.0 ± 0.1 (TC15), and 6.0 ± 0.1 (TC20) (Table S1). At the end of the experiment, the differences in pH were significant among the three treatments. Organic matter, organic carbon, estimated nitrogen release, and available phosphorus also increased with increasing compost amendment. There were statistical differences between the 10 % and 20 % treatments in all cases while the 15 % treatment was statistically similar to the 10 % treatment in one case (available P), was similar to the 20 % treatment in two cases (organic matter and estimated nitrogen release), and similar to both for organic carbon. The total exchange capacity decreased significantly from 10 % to 20 % compost treatments, while in contrast,

percent base saturation of cations Ca and Na increased significantly. In addition, ammonium-N, extractable P, Na, Fe, Mn, and percent base saturation of cations Mg and K had consistent significant differences only between the TC10 and TC20 treatments (Table S1).

The metal(loid) content of the unamended tailings was 2332, 3219, and 2644 mg kg⁻¹ Zn, As, and Pb, respectively, while the compost had negligible amounts of metal(loid)s (Table S1). The addition of compost diluted the metal(loid) content of tailings in TC10 by 9.6 ± 2.7 %, in TC15 by 10.2 ± 1.2 %, and in TC20 by 15.6 ± 2.0 % (measured in the unseeded control pots; Table S1). Metal(loid) concentrations measured at the end of the experiment were similar to the initial values for As and Pb for both the control unplanted and experimental planted pots, but the final concentrations of Zn were lower by an additional 49 % (TC10), 30 % (TC15), and 16 % (TC20) in the experimental pots (Table S1). It is likely that the loss of Zn from the system was due to leaching as Zn concentrations were similarly lower in the unseeded control pots at the end of the experiment (Table S1). Notably, for both planted and unplanted pots, final extractable Zn (available) concentrations were similar in TC15 and TC20 and significantly lower in TC10 (Table S1).

3.2. Treatment effects on plant properties and metal uptake

Zinc accumulated significantly more in above ground shoot and leaf organs in the order TC15 > TC10 > TC20 (Fig. 1). In particular, leaf accumulation of Zn in the TC15 treatment exceeded the Zn hyper-accumulation threshold (3000 mg kg⁻¹; van der Ent et al., 2013). In contrast, there was no difference in accumulation of As into above-ground plant organs for any treatment. For Pb, the only significant difference was that the plants in TC20 treatment accumulated significantly more than the TC10 treatment.

In terms of plant phenotypic metrics, there was a trend of increasing plant height and number and length of branches with increasing compost amendment, but the differences were significant only for plant height (Fig. S5). Interestingly, the plants from TC15 treatment had the highest qualitative score for visible symptoms of tailings toxicity on the leaves and this difference was significant (Fig. S5).

3.3. Differences in microbial community composition across treatments

In the absence of plants, bacterial/archaeal richness (number of ASVs) in TO and TC10 were very low both at initial and final time point (richness <25; Fig. S6A). Initial richness was higher in the TC15 and TC20 control pots and richness increased further in these two treatments at the end of the experiment. The initial bacterial/archaeal phyla composition in the TC15 and TC20 treatments was similar to the compost material (Fig. S6B).

In the presence of plants, by the end of the experiment, bacterial/archaeal richness as well as Shannon diversity increased significantly in bulk soil as a function of compost amendment (Fig. 2A). For rhizosphere soil, the T10 and T15 treatments were similar in richness and Shannon diversity with a significant increase occurring in the T20 treatment. A significantly positive rhizosphere vs. bulk samples effect was observed only in the TC10 treatment. Community ordination analysis identified treatment as explaining 44 % of the variation in the bacterial/archaeal community composition ($p = 0.001$) (Fig. 2B).

DESeq2 LRT analysis across the three treatments revealed 32 genera that were differentially abundant ($p\text{-adj} \leq 0.05$) in rhizosphere samples (Fig. S7). For TC10 samples, *Hydrotalea* (5.09 %), *Allicyclobacillus* (3.81 %), and *Dyella* (2.43 %) had the highest abundance. Two of the three most abundant genera in the TC15 treatment (*Hydrotalea*, 9.55 %; *Allicyclobacillus*, 6.23 %; and *Pseudolabrys*, 4.16 %) were the same as for the TC10 treatment. But the three most abundant genera in the TC20 treatment (*Acidibacter*, 2.15 %; *Sporocytophaga*, 1.45 %; and *Steroidobacter*, 1.3 %) were different from both the TC10 and TC15 treatments. We compared the total relative abundance of the three most abundant genera in each treatment. For TC10, the abundance totaled to

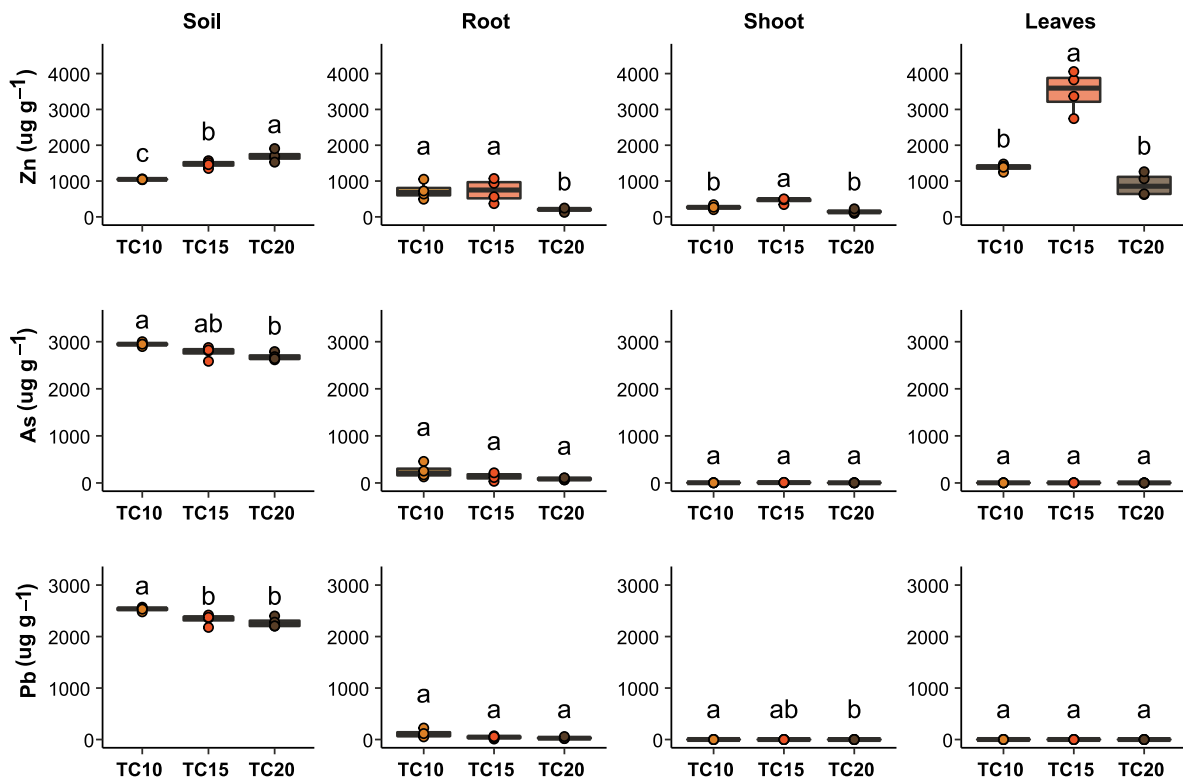


Fig. 1. Metal(loid)s concentration in soils and *A. lentiformis* root, shoot, and leaf samples. Each box represents the inter-quartile range of the data, with the median indicated by the horizontal line. Circles represent the individual datapoints. Different letters indicate statistically significant differences at $p \leq 0.05$ (ANOVA with Tukey's HSD test).

11.33 %, for TC15, 19.94 %, and TC20, 4.9 % suggesting that community selection pressures were quite different in these treatments. These data are interesting in light of the fact that the TC15 treatment had the highest Zn concentrations in plant leaves (Fig. 1) and the highest score for visible stress symptoms on leaves (Fig. S5).

3.4. Significantly expressed *A. lentiformis* root genes across treatments

Using DESeq2, 190 root genes ($p\text{-adj} \leq 0.05$) were significantly expressed across the three treatments (Fig. 3). A PCA plot of significantly expressed gene profiles highlights the separation of the gene expression profiles of the treatments with the first two PCA axes explaining 83 % of the variation in gene expression (PC1, 74.3 % and PC2, 8.4 %; Fig. S8). Forty-eight of the 190 genes decreased in gene abundance with increasing compost amendment (Fig. 3). These genes were associated with the functions in auxin signaling, biosynthetic processes, calcium/calmodulin binding, defense responses, cell wall organization, development, ion channels, metabolic processes, metal ion binding, oxidative stress, transcription regulation, RNA modification, and transmembrane transport (Fig. 3). The specific functions of some of these genes include cellulose biosynthesis (*CESA3*; cellulose synthase A catalytic subunit 3), cell wall organization (*GPDL3*; glycerophosphodiesterase-like), plant defense against bacterium (*IRE1A*; serine/threonine-protein kinase/endoribonuclease and *TET8*; tetraspanin-8) and inflorescence development (*YODA*; mitogen-activated protein kinase (MAPK) kinase kinase; Fig. 3). In contrast, 15 genes increased in abundance with increasing compost amendments. Some of these genes included *ZIP4*, *IP5P6*, *FAS2*, *ATCA1*, and *XTH32* which are involved in transmembrane transport, auxin signaling, DNA recombination and repair, one-carbon metabolic process, and cell wall biogenesis, respectively (Fig. 3).

Notably, the TC15 treatment had 19 of the highest abundance genes and 106 of the lowest abundance genes (Fig. 3). Some of the 19 upregulated genes in TC15 are involved in abscisic acid (ABA) signaling

(*AI5L2*, *MFT*, *P2CO3*), auxin signaling (*MAX*, *WAT1*), transcription regulation (*DPB*, *NFYB3*), and RNA modification (*PP360*, *PPR8*). With the exception of auxin signaling and ion channel gene categories, the downregulated genes in TC15 were associated with the remaining of the 23 gene categories (Fig. 3).

3.5. Zn accumulation associations with abiotic and biotic variables

Due to the variable accumulation of Zn in plant leaves across treatments, a PLS analysis was performed to examine associations with abiotic and biotic variables. The first two PLS components explained 86 % of the variations in Zn leaf concentration (Fig. S9 and S10). All the explanatory variables explained 52 % and 34 % of variance for the first and the second PLS component, respectively, and Zn leaf concentrations explained 49 % (PLS 1) and 28 % (PLS 2) of the variance. The significance of each explanatory variable in the PLS model was assessed based on the Variable Importance for Projection (VIP) > 1.0 criterion (Table S2). Accordingly, 42 out of 268 variables were considered important for Zn leaf accumulation. The relative contribution of each variable in explaining the differences in Zn accumulation in leaves was assessed by the absolute values of the standard regression coefficients (Fig. 4).

Out of the 42 variables identified as explanatory, 16 and 26 variables had a positive and negative association with Zn accumulation, respectively. The variables were a combination of Zn shoot concentration, TC15 and TC20 treatments, leaf stress symptom score, 10 bacterial genera, and 28 root genes. The top five variables that had strong positive associations in our model included: Zn shoot concentration, the TC15 treatment, *MAX1* and *MFT* genes, and the bacterial genus, *Hydrotalea* (Fig. 4). Notably, the genes that associated positively with Zn accumulation all had higher gene abundance in TC15 treatment in comparison to the TC10 and TC15 treatments (Fig. S11). Also, the leaf stress symptoms score was identified as an important variable that explained

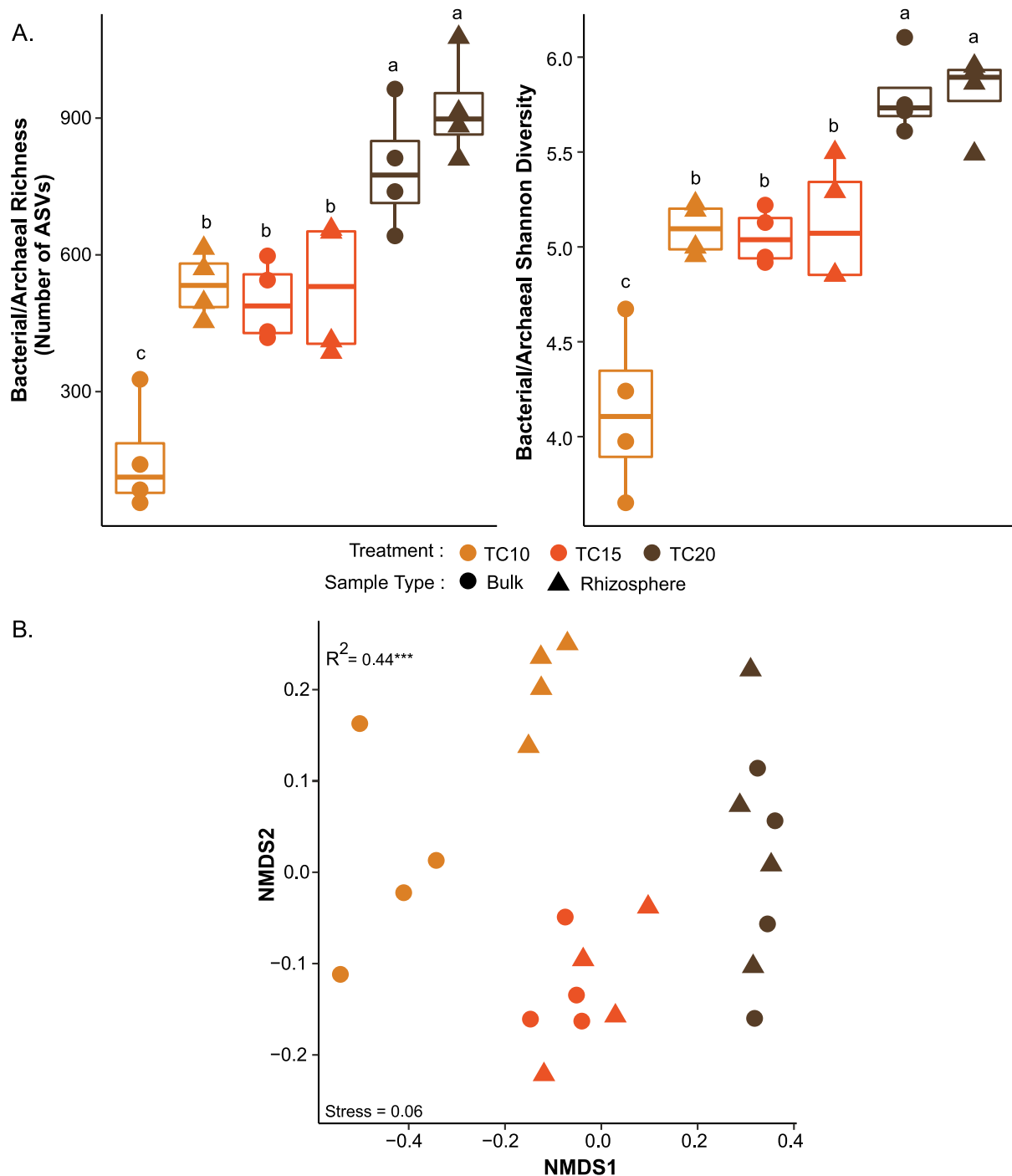


Fig. 2. Microbial diversity in the 10 % (TC10), 15 % (TC15), and 20 % (TC20) compost treatments. (A) Bacterial/archaeal richness and Shannon diversity index for bulk (circle) and rhizosphere (triangle) samples. The boxes represent the inter-quartile range of the data, the median is indicated by the horizontal line. Significant differences are represented by different letters (Kruskal-Wallis test). (B) Nonmetric multidimensional scaling (NMDS) ordination plot of microbial community structure. R^2 represents the variation in the microbial community composition explained by treatment (p -value = 0.001; PERMANOVA).

Zn accumulation, with a strong positive association. On the other hand, three plant genes (*BABL*, *MTHR2*, *U86A1*), the TC20 treatment, and the bacterial genus *Bauldia* were strong negative predictors. All 18 genes except *IPMKB* that had negative associations with Zn accumulation had the lowest gene abundance in TC15 across the three treatments (Fig. S12).

4. Discussion

4.1. Plant metal uptake and growth is influenced by different compost treatments

In our study, neither As nor Pb accumulated substantially into the aboveground plant organs. In contrast, Zn accumulation into *A. lentiformis* leaves exceeded 500 mg kg^{-1} (domestic animal toxicity limit (DATL) limit) in the TC10 and TC20 treatments and for the TC15 treatment, Zn was hyperaccumulated, as defined by [van der Ent et al.](#)

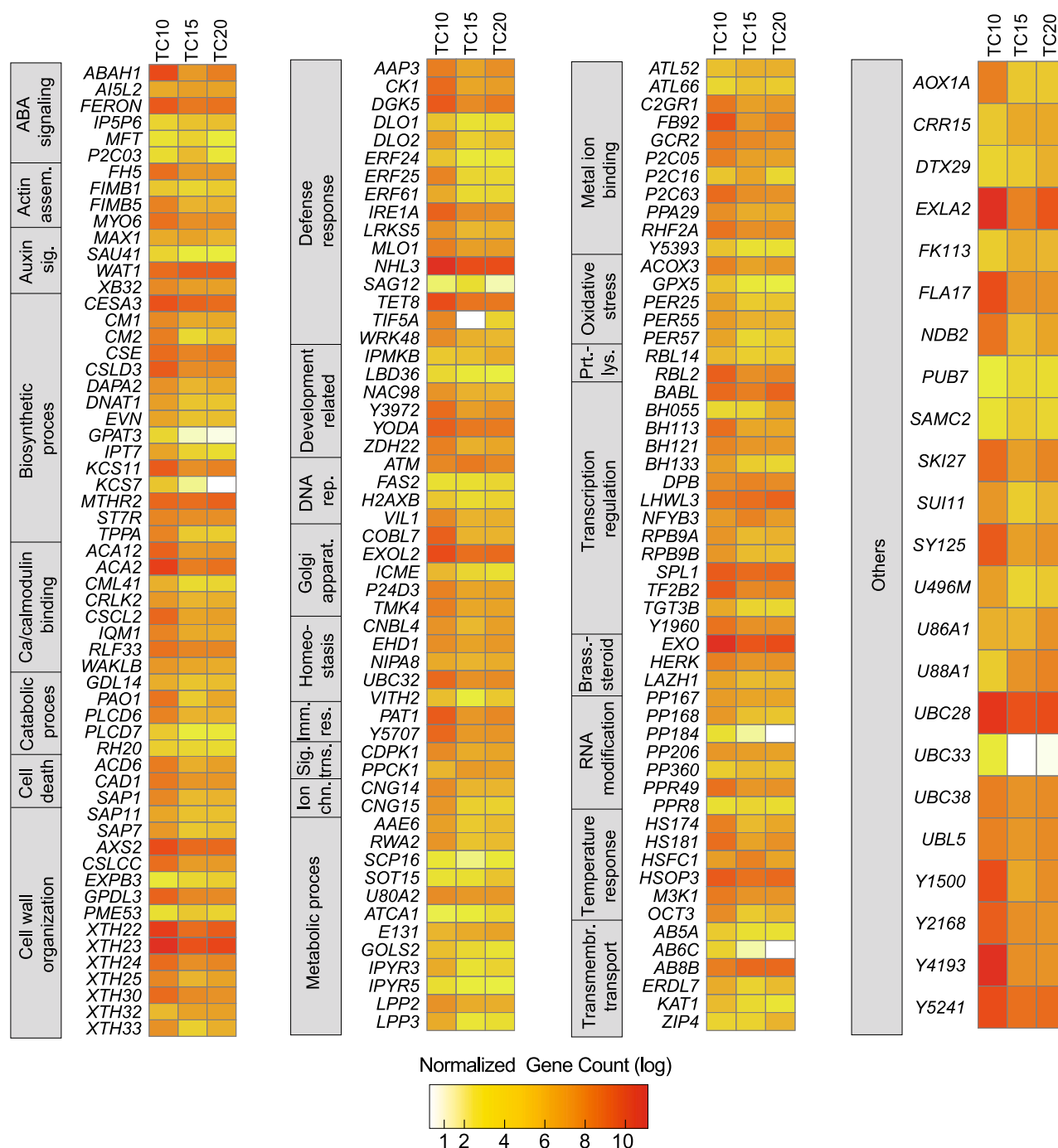


Fig. 3. Abundance of transcripts expressed in *Atriplex lentiformis* roots in the 10 % (TC10), 15 % (TC15), and 20 % (TC20) compost treatments. Normalized gene (log) count of genes that were significantly different in gene expression according to the likelihood ratio test of DESeq2 analysis are shown ($p\text{-adj} \leq 0.05$). The genes are ordered as per their putative function. Actin assem. = Actin filament assembly; Auxin sig. = Auxin signaling; DNA rep. = DNA repair/replication; Golgi apparat. = Golgi apparatus; Imm. Res. = Immune response; Sig. trns. = Intracellular signal transduction; Ion chn. = Ion channel; Prt.-lys. = Proteolysis; Brass.-steroid = Brassinosteroid response; Transmembr. transport = Transmembrane transport.

(2013), to levels exceeding $>3000 \text{ mg kg}^{-1}$. This is in contrast to a previous study where although Zn concentrations in *A. lentiformis* shoots surpassed the DATL limit, they did not exceed the hyperaccumulation threshold (Solís-Domínguez et al., 2012). An important difference between these studies is that Solís-Domínguez et al. (2012) combined both shoots and leaves in their analysis while in the present study shoots and leaves (which have a much higher Zn concentration than shoots; Fig. 1) were analyzed separately. Despite the increased Zn accumulation in TC15, available Zn concentrations, as estimated by the Mehlich-III extractable method, were not higher in TC15 soils. In fact, total Zn

and available Zn showed a similar trend and both increased with increasing compost levels. Similar Zn hyperaccumulation has been previously reported in a *Arabidopsis halleri* population despite low concentrations of bioavailable Zn in the soils (Dietrich et al., 2021; Kushwaha et al., 2022). Thus, these findings demonstrate that Zn accumulation in *A. lentiformis* varied independent of any of the soil chemistry variables, including total metal concentrations and available Zn concentrations in soil. These results suggest that the Zn accumulation patterns in *A. lentiformis* were more driven by plant physiology rather than soil chemical variables as has been previously reported in *A. halleri*

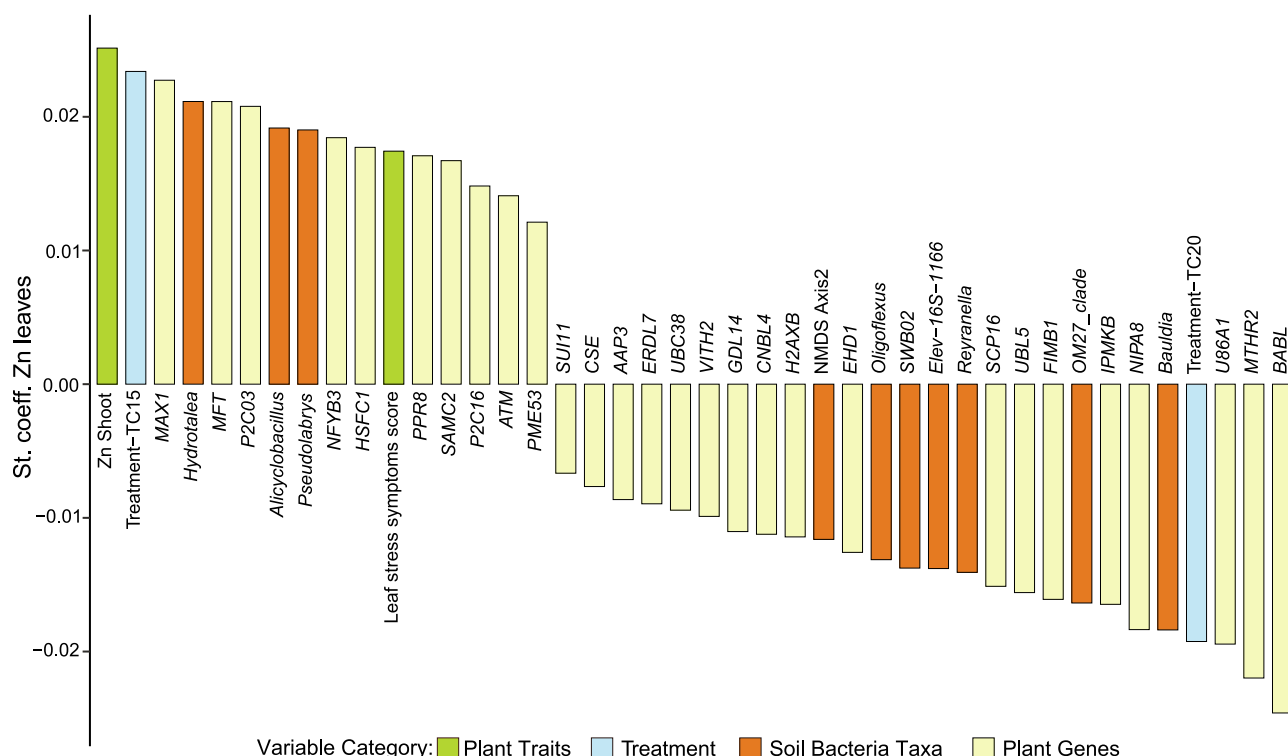


Fig. 4. Abiotic and biotic drivers of Zn accumulation as determined by partial least square (PLS) regression analysis. The relevant explanatory variables are arranged in the descending order based on their standardized regression coefficients (St. coeff.; variables with Variable Importance for Projection (VIP) > 1.0).

(Dietrich et al., 2021).

Increasing the pH of acidic soils has been shown to maximize nutrient availability and is considered optimal for plant growth (Neina, 2019). The addition of compost to the IKMHSS mine tailings increased the pH to circumneutral values. As might be expected, this was reflected in trends of increasing plant height and branch length with increasing compost amendment. Thus, it was unexpected that the TC15 plants developed the most visible stress symptoms, including chlorosis, mottling, and necrosis of foliage. We hypothesize that this is due to the Zn hyperaccumulation that was observed in TC15. In fact, stress response due to Zn accumulation in plant foliage has been previously reported (Hermle et al., 2007; Babst-Kostecka et al., 2016, 2018). The allocation of excess Zn to chloroplasts and other sensitive sites in the cell cytoplasm causes increased oxidative stress, which is a typical consequence of Zn over-accumulation in plants (Gratão et al., 2005). An overload of defenses against oxidative stress can lead to increased photo-oxidative stress, accelerated cell senescence and hypersensitive response-like (HR-like) mechanism (Günthardt-Goerg and Vollenweider, 2007), with the latter mechanism primarily known as a defense against pathogens (Fritig et al., 2007) but also shown to be triggered by abiotic stress factors.

4.2. Compost amendment influence on the microbiome and implications for sustained plant growth

We examined the microbial community after 11 weeks of plant growth. In terms of plant metrics, there was little difference among treatments; plants were similar across treatments with respect to number of branches and length of longest branch. The only difference was that the TC10 treatment had a significantly lower plant height than the TC20 treatment (although it was not different than the TC15 treatment). In terms of the substrate microbiome, both richness and community composition were similar for bulk and rhizosphere samples in the TC15 and TC20 treatments. These results mirror a field trial at IKMHSS (Honeker et al., 2019) and are similar to a previous study showing that

bacterial richness of bulk and rhizosphere in plant-sustaining soils is similar (Mosqueira et al., 2019). Other studies have also reported similar richness in bulk and rhizosphere soils at metal-polluted sites with well-established vegetation cover (Gao et al., 2021; Sun et al., 2021a; Kushwaha et al., 2022). Overall, these results suggest that the similar bulk and rhizosphere microbial diversity patterns found in the TC15 and TC20 treatments is an indicator of compost-amended tailings' ability to support plant growth. In contrast, the TC10 treatment exhibited a rhizosphere microbial community that had higher richness than the bulk community. This could be attributed to plant-mediated modifications of the rhizosphere microbial community to select and support plant-sustaining microbes at lower pH (< 4) (Honeker et al., 2019), which seems to have been an effective strategy since the plant metrics in the TC10 were largely similar to those in the TC15 and TC20 treatments. These results also suggest that the bulk community in the TC10 treatment may not have the requisite diversity to support plant growth over the long-term. This is supported by results from a 5-year field study at the IKMHSS in which the percent canopy cover trended downward from 30 % to 12 % in the TC10 treatment over the course of the study while the canopy cover in the TC15 and TC20 treatments was maintained at steady levels (Gil-Loaiza et al., 2016; unpublished data). In summary, our results highlight that different compost treatments influence the rhizosphere microbiome. However, to have a better understanding of the effect of the root compartment on microbial diversity, structure, and composition, future studies should include rhizoplane analyses.

4.3. Zn accumulation in TC15 treatment is influenced by specific microbial taxa

The genus *Hydrotalea* had the highest relative abundance (10 %) in the TC15 treatment, followed by *Alicyclobacillus* (6 %) and *Pseudolabrys* (4 %) and these microbial taxa are strongly associated with metal contamination. *Hydrotalea* and *Pseudolabrys* have been previously reported in high abundance in a Brazilian acid mine drainage system and in soils contaminated with cadmium (Cd) during phytostabilization of a

metal-tolerant plant species, *Miscanthus spp.*, respectively (Lemos et al., 2019; Chen et al., 2021). Moreover, heavy metal tolerance has been reported in a strain of *Alicyclobacillus*, isolated from an As-rich hot spring (Aulitto et al., 2021). Previous studies have demonstrated increased abundance of *Alicyclobacillus*, when autotrophic and heterotrophic microbial mixtures were applied to assess their phytoextraction and bioremediation efficiency in Cd contaminated soils (Hao et al., 2019; Xu et al., 2020). The presence of these rhizosphere taxa in high abundance in the TC15 treatment and their known associations with Cd hyper-accumulation suggest that these microorganisms may also stimulate Zn uptake by *A. lentiformis* in TC15 treatment. Overall, these findings highlight the importance of further research to elucidate the mechanisms by which plant-associated microbial taxa alleviate metal stress and/or assist in phytoextraction of different metal(loid)s by plants.

4.4. Plant root gene expression under different of levels of metal contamination

Results from this study show that plant responses as a function of compost amendment resulted in differential gene expression changes related to ABA signaling, auxin signaling, defense responses, responses to salinity and temperature, oxidative stress, and transcription regulation. First, we will discuss examples of calcium/calmodulin binding proteins, serine/threonine-protein kinases, ethylene response factors, and oxidative stress genes that were up-regulated in the TC10 treatment. Calmodulin-like proteins and Ca^{2+} /calmodulin-dependent protein kinases act as Ca^{2+} sensors and regulate a variety of proteins that respond to environmental stressors (Zeng et al., 2015). In fact, upregulation of these genes has been associated with tolerance to salt stress in rice (Campo et al., 2014). Similarly, high salt conditions can activate Ca^{2+} and MAPK signaling pathways via serine/threonine protein kinase PP3C (de Zélicourt et al., 2016). Thus, the overexpression of calmodulin-like proteins (CML14, CRLK2) and serine/threonine protein kinases (Y5707, IREIA, Y1960) in the TC10 treatment suggests that *A. lentiformis* plants are under increased stress in this treatment.

ERF genes play a role in plant growth and development processes as well as stress resistance and defense response (Gu et al., 2017). In previous studies, ERF transcription factors (AP2/ERF109 and AP2/ERF020) were upregulated in *Arabidopsis* and rice plants, respectively, under Cd stress (Weber et al., 2006; He et al., 2015). Also, ethylene triggers the ERF gene signaling pathway which is known for its defense response against plant pathogens, such as hypersensitive responses that control the spread of pathogens (Kacperska, 1997). Given that the plants in the TC10 treatment were subjected to the lowest pH and highest metal concentrations, upregulation of *ERF25* is not surprising. Similarly, *GPX* genes which encode glutathione peroxidases that are induced by oxidative stress, including *GPX5*, are important for detoxification of hydrogen peroxides (Mittler, 2002; Bela et al., 2015). Further, Koh et al. (2007) demonstrated that *GPX5* has a unique dimerization pattern that enables it to interact with Cd^{2+} ions—indicating a direct interaction between metal stress and *GPX5*. In this study, *GPX5* was upregulated in the TC10 treatment. Unexpectedly, in the TC15 treatment, where plants showed visible signs of stress, none of the differentially expressed oxidative stress genes were upregulated. Taken together, these findings suggest that there may be different strategies that plants in the three compost treatments use to mitigate metal stress.

In the TC15 treatment, a set of genes was overexpressed that have strong association with metals including: more axillary growth1 (*MAX1*), protein phosphatase 2Cs (*P2C03*), nuclear transcription factor Y subunit (*NFYB3*), and heat shock transcription factor (*HSFC1*). *MAX1*-like genes are involved in the biosynthesis of strigolactones and their homologs have been reported to respond to heavy metals (Marzec et al., 2020). Further, Marzec et al. (2020) reported that highly co-expressed genes with *MAX1* were involved in Cd response in rice as well as regulation of heavy metal transport/detoxification. Also, overexpression of *MAX1* gene altered the rhizosphere bacterial and fungal microbiome

associated with soybean (Liu et al., 2020). Thus, strigolactone biosynthesis could be exploited to place selective pressure on the soil microbiome and thereby, indirectly enhance the ability of plants to accumulate metals (Mostofa et al., 2018). By inference, this suggests that the high abundance of *Alicyclobacillus*, *Hydrothalea*, and *Pseudolabrys* and overexpression of *MAX1* gene in TC15 plants has the potential to increase Zn uptake in *A. lentiformis*.

Abscissic acid (ABA) signaling is an essential plant hormone pathway that responds to various abiotic stressors like cold, drought, salinity, heat, and metal toxicity. Protein phosphatase 2Cs (*P2C03*) have been reported to negatively regulate the ABA signaling pathway (Saini et al., 2020). As ABA has been reported to alleviate metal uptake and accumulation of Zn in grapevine (Song et al., 2019), the overexpression of *P2C03* in TC15 plants suggests that inhibition of ABA signaling may have resulted in Zn accumulation in *A. lentiformis*. Similarly, a NTFY subunit (*11NF-YC6*) has been shown to enhance tolerance to Cd stress in *A. thaliana* (Ni et al., 2023). Interestingly, HSFs are not only associated with genes that respond to high temperatures, but it also regulates genes associated with drought and heavy metals (Rehman et al., 2021). Consequently, overexpression of *HSFA4a* conferred Cd tolerance in wheat and rice (Shim et al., 2009).

Out of the 190 genes that were significantly expressed across the three treatments, only 15 genes increased in abundance with increasing compost levels. One example of a gene that was upregulated in 20 % compost treatment is zinc/iron regulated transporter proteins from ZIP family that are involved in metal translocation within plant organs (Sun et al., 2022). In fact, in *A. thaliana*, ZIP4 protein is associated with translocation of different heavy metals (Wintz et al., 2003). However, up-regulation of *ZIP4* in the present study did not result in enhanced translocation and accumulation of metal(loid)s in the aboveground plant organs in TC20 treatment. Accordingly, these results re-emphasize that *A. lentiformis* in the three compost treatments may use different strategies to mitigate metal stress. Lastly, it is important to highlight that the *A. lentiformis* transcripts in our study were annotated against the model plant species with robust gene annotation, *A. thaliana*—a species not closely related to *A. lentiformis* phylogenetically. Further, BLASTX analysis of *A. lentiformis* transcripts against a phylogenetically close species, *Chenopodium quinoa* did not result in a significantly higher gene homology (data not shown). As a result, our findings on genes associated with heavy metal stress and detoxification in *A. lentiformis* may be incomplete and further research, including whole genome assembly of *A. lentiformis* with functional gene annotation, is needed to understand the gene pathways that are involved in alleviating metal stress in this species. Additionally, future studies should observe the composition and organization of the cell wall in *A. lentiformis* root cells using scanning or transmission electron microscopy as organization of the cell wall has been associated with tolerance to metal accumulation (Corso and García de la Torre, 2020; Wu et al., 2020).

4.5. Significance of assembling a de novo A. lentiformis transcriptome

In this study, we carried out a *de novo* transcriptome assembly of *A. lentiformis* to identify genes in response to metal-stress during growth of *A. lentiformis* under different levels of metal contamination. Although *A. lentiformis* has been extensively studied for its ability to grow under stressed conditions (Bose et al., 2015; Eissa, 2017; Eissa and Abeer, 2019), its genome has not previously been sequenced. Therefore, assembling *A. lentiformis* transcriptome for the first time is of significance for several reasons. First, our reference *A. lentiformis* transcriptome has enabled us to explain genetically why *A. lentiformis* is adapted for extreme environments. Second, this *A. lentiformis* will allow the transcriptome to be used for comparative analyses among other phytoremediating species as well as to study evolutionary conservation of metal stress genes across different species. Third, *A. lentiformis* with its C4 photosynthetic mechanism is of considerable importance in studying the evolution of C4 photosynthesis as C4 plants are much more water

efficient than plants that only use C3 (Zhu and Meinzer, 1999). Use of C4 apparatus has been studied in the context of salt stress in *A. lentiformis* and other species (Abogadallah, 2010; Sun et al., 2021b) but with our assembly, we can now study *A. lentiformis* for heavy metals stress and tolerance genes at the genome level.

5. Conclusions

In this study, we highlight the associations between the soil microbial community, metal uptake, and plant gene expression in *A. lentiformis* grown under three levels of compost-amended, acidic, metalliferous-mine tailings. Our results show that Zn accumulation in leaves of *A. lentiformis* varied with compost treatments and was the highest in the intermediate compost treatment (15 %; TC15). Results suggest that Zn accumulation is predominantly governed by the plant and soil microbiome and not soil chemistry. We have identified three microbial taxa associated with responding to metal stress and uptake that were also strongly associated with Zn accumulation in *A. lentiformis*. Furthermore, this study is the first to build a *de novo* *A. lentiformis* transcriptome assembly and demonstrate *A. lentiformis* root gene expression in three different compost treatments. Root genes associated with ABA and auxin signaling, biosynthetic processes, calcium/calmodulin binding, defense response, cell wall organization, development, ion channels, metabolic processes, metal ion binding, oxidative stress, transcription regulation, RNA modification, and transmembrane transport were identified in *A. lentiformis*. Moreover, results show that the changes in root gene expression were not primarily driven by the increasing levels of compost. For example, there were 106 genes that were down-regulated in TC15. In contrast, 10 genes that were up-regulated in TC15 strongly associated with Zn accumulation. Thus, we highlight that *A. lentiformis* plants grown under different metal stress levels respond differently using different plant gene pathways to alleviate the stress. Future work should focus on: i) investigating how microbial taxa affect Zn metal uptake in *A. lentiformis* under different organic amendments, ii) gaining a mechanistic understanding of different strategies employed by *A. lentiformis* under different metal stress. Such findings will ultimately help develop better phytoremediation management strategies for metal-contaminated sites.

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CRedit authorship contribution statement

Priyanka Kushwaha: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Funding acquisition. **Alexandria Tran:** Investigation, Formal analysis. **Diego Quintero:** Investigation, Formal analysis. **Miranda Song:** Investigation, Formal analysis, Visualization. **Qi Yu:** Investigation. **Ruth Yu:** Investigation, Writing – review & editing. **Michael Downes:** Investigation. **Ronald M. Evans:** Resources, Funding acquisition. **Alicja Babst-Kostecka:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Julian I. Schroeder:** Supervision, Project administration, Methodology, Writing – review & editing, Funding acquisition. **Raina M. Maier:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Sequences have been submitted to NCBI database with metadata

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