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Extraction Potential of *Lolium perenne L.* (Perennial Rye Grass) for Metals in Landfill Soil: Its Tolerance and Defense Strategies

Mmatsheko Kgaladi Leah Masotla [1], Funzani Asnath Melato [1,*] and Ntebogeng Sharon Mokgalaka-Fleischmann [1,2]

[1] Department of Chemistry, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa
[2] Mamelodi Campus, University of Pretoria, Private Bag X20, Pretoria 0028, South Africa
* Correspondence: melatofa@tut.ac.za; Tel.:+27-12-382-6395

Abstract: Landfill sites open and close frequently throughout the world, taking over a significant amount of land and leaving it contaminated and unavailable to the surrounding population for use. Different forms of remediation methods have been employed to rehabilitate contaminated land to a state that poses less of a threat to the environment. Phytoremediation is one of the remediation techniques that has proven to be effective, economical and easier to implement compared to other methods. The main aim of this study was to explore the potential use of *Lolium perenne* L. to remediate and restore metal-contaminated landfill soil and determine its stress tolerance mechanism(s). The metal uptake, determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and inductively coupled plasma-mass spectroscopy (ICP-MS), revealed that *Lolium perenne* accumulate a higher amount of metals in the roots than in leaves, which was further confirmed by the translocation factor (TF) values of all of the metals that were below 1, ranging between 0.2 and 0.8, while Cu, Cr and Pb had a bioaccumulation factor (BCF) > 1. This confirms that *L. perenne* is capable of absorbing metals into the root matrix but might restrict further movement into other parts of the plant as a defense mechanism against metal toxicity. In response to metal-induced stress, *L. perenne* displayed an increase in enzyme activity of superoxide dismutase, glutathione S-transferase, peroxidase and amylases in plants grown in landfill soil. Peroxidases displayed the highest level of enzyme activity, while total amylolytic activity had the most significant increase in activity over time. Although not a hyperaccumulator, *L. perenne* is a potential candidate for the phytoremediation of landfill soil and the phytostabilization of Cu, Cr and Pb.

Keywords: phytoremediation; landfill soil; antioxidant enzymes; bioconcentration factor (BCF); translocation factor (TF); *Lolium perenne*

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1. Introduction

Landfilling is considered to be a cost-effective method of disposal for solid waste across the globe [1]. According to the World Bank [2], the world generates approximately 2.01 billion tons of waste annually, with less than 70% of the waste being managed in an environmentally safe manner. Global waste is expected to increase to 3.4 billion tons over the next 30 years, which is a clear indication of a need for increased remedial action. However, mismanagement of solid waste in the process of disposal or containment can result in it becoming a breeding ground for disease vectors and pests, posing a risk to human health [3]. Furthermore, leachate resulting from the seepage of rainwater through contaminated soil is the main source of groundwater pollution. Once contaminated, groundwater quality cannot be fully restored [4]. Landfill leachate contains a wide range of pollutants; this includes dissolved organic matter; inorganic compounds such as Ca, Mg and chlorides; and heavy metals, such as Cd, Cu, Ni, Pb and Zn, among others, that may have detrimental effects on human health, as well as the environment [4]. The nature and concentration of the contaminants found in the leachate depend on the composition of the landfill soil, the type of

waste being deposited at the landfill and the climate, among other things [5]. The mobility of the heavy metals found in leachate allows them to migrate away from the landfill site to other sites and water reserves, posing a major health risk for surrounding communities [6]. Once a landfill site has exhausted its lifespan, it is closed, and this presents a need for the containment of any potential contamination of surrounding environments. Post-closure, landfills require rehabilitation to reduce their potential impact on their surroundings. Most rehabilitation methods are very costly, limiting the options that are available for use at landfill sites, as they usually cover a large amount of land [7].

Phytoremediation presents a cost-effective, nonintrusive and ecologically benign method for the removal of contaminants from the environment [8]. This is a process through which potentially toxic substances are removed from the environment and transformed into metabolites or less harmful end-products by plants. Phytoextraction and phytostabilization are two mechanisms through which plants are able to take up contaminants from their growth medium and localize them in different organs [9]. When a plant is able to accumulate a specific metal in quantities that are in excess of 1% of the plant's dry mass and continue to thrive, it is considered to be a metal hyperaccumulator [10,11].

Lolium perenne L., commonly known as perennial ryegrass, belongs to the plant family Poaceae. This is one of the 400 plant families that are known to be metal hyperaccumulators [12]; it is also known to be one of the 11 dominating families in this group. Although *L. perenne* is not a metal hyperaccumulator, its tolerance for some metals, hardiness under unfavourable conditions, and rapid establishment which allows quick ground cover once planted, make it a great candidate for phytoremediation [13].

Metals such as Co, Cu, Fe, Mn, Mo, Ni and Zn are essential for certain biochemical processes in plants and are thus referred to as essential micronutrients or trace elements [14]. These metals are only required in small quantities and can be detrimental to plant life at excessive levels [15]. Stunted development and altered metabolic and physiological pathways as a result of interference by these metals are some of the recognized biochemical indicators of metal toxicity in plants [16].

Plants have been reported to experience oxidative stress as a result of excessive metals and unfavourable circumstances. Reactive oxygen species (ROS), which can harm cells and alter metabolic pathways, are produced in greater quantities as a result of oxidative stress [17]. Although excessive quantities of ROS can be harmful to plants, ROS are necessary under normal conditions. They serve as secondary messengers for several cellular functions, such as plant stress tolerance. Whether ROS fulfill their function as messengers or negatively affect cellular activity depends on the balance between ROS generation and scavenging in the cells [18]. Research has shown that the presence of some metals in excessive quantities in plants triggers antioxidant enzyme activity in affected cells [19]. Antioxidant enzymes are able to scavenge ROS, thus reducing or even eliminating their impact on plant health [20].

Several plants, including grasses, have been the focal point of phytoremediation research because of their ability to hyperaccumulate toxic metals and restore surrounding environments. However, information regarding plant species suitable for the rehabilitation of landfills is limited.

The phytoremediation potential of *L. perenne* for the rehabilitation and reclamation of landfills was explored in this study. The ability of *L. perenne* to bioaccumulate metals from the landfill soil and the use of different enzymes to mitigate the resulting stress on plant metabolic pathways were also investigated. Over and above this, the ability of the plant to thrive under these conditions was central to it being confirmed as a worthy candidate for phytoremediation.

2. Materials and Methods

2.1. Pot Trial

Landfill soil was collected from three different points at the Onderstepoort landfill site (GPS coordinates: −25.65135, 28.15708; Pretoria, Gauteng, South Africa). Potting soil

purchased from a local nursery was used as the control soil. *L. perenne* seeds purchased from Agricol (Pretoria, Gauteng, South Africa) were germinated on seedling trays using potting soil in a greenhouse under natural conditions. The seedlings were allowed to grow for a month before being transplanted into potting bags containing landfill soil. Three seedlings were placed per bag in 2 L potting bags, with a total of 36 bags for the landfill soil and 36 for the control soil. The plants were allowed to grow for six weeks before being harvested, and a set of three plants per pot were harvested weekly from each soil for a period of 12 weeks. The plants were washed, separated into shoots and roots, dipped in liquid nitrogen and then freeze-dried for 78 h. The plant material was then ground in preparation for further analysis.

2.2. pH and Conductivity

Samples of landfill soil and control soil were collected prior to the phytoremediation pot trial and during each harvest. The soil samples were air-dried and then ground to a fine powder and sieved with a 2 mm sieve. Using a 1:2 dilution ratio, 5 g of air-dried, ground landfill soil was dissolved in double-deionized water. The solution was shaken on a platform shaker for 18 h at 200 rpm and centrifuged for 10 min at -1792 relative centrifugal force (RCF). The supernatant was then filtered; thereafter, pH and electrical conductivity readings were taken using the XS Multiparameter Professional Bench Meter (XS Instruments, Carpi, MO, Italy).

2.3. Metal Analysis

2.3.1. Acid-Assisted Microwave Digestion of Samples

To establish the distribution of metals in the tissues of *L. perenne* after exposure to landfill soil, roots and leaves from the harvested plants were analyzed. The baseline metal concentrations of the landfill and control soil along with the residual metal content in the soil collected at each harvest were also determined to assess the depletion of these metals in the soil and their accumulation in plant parts over the experimental period. Approximately 0.25 g ground leaves, roots and soil were weighed into microwave vessels, to which 5 mL of Suprapur 65% (*v*/*v*) HNO_3 (Merck, Darmstadt, Germany) and 1 mL HF (Merck) were added under a fume hood. This was followed by digestion using a MARS Xpress microwave digestion system (CEM Cooperation, Matthews, NC, USA) at 190 °C and 1200 W for 20 min. A second digestion stage was carried out for the roots and soil by adding 1 mL freshly prepared 7% (*m*/*v*) boric acid (Merck) to the sample and digesting at 170 °C and 1200 W for 10 min.

2.3.2. Determination of the Concentration of Metals in Soil, Leaves and Roots

A Spectro ARCOS® ICP-OES (Spectro Instruments, Kleve, Germany) was used to determine the concentrations of Cu, Cr, Mn, Pb and Zn in soil, leaves and roots. A 1000 ppm ICP grade multi-element stock standard solution (Fluka Chemie AG, Buchs, Switzerland) was used and diluted to prepare standards for calibrating the instrument. The concentration of Cr in the control leaves and roots harvested in week seven was below the detection limit of the ICP-OES, and it was subsequently analyzed using ICP-MS (ELAN DRC-e, Perkin Elmer, Shelton, CT, USA). The operating parameters of the instruments are provided in Table 1.

Table 1. Operating parameters of the ICP-OES and ICP-MS.

Operating Parameter	Spectro ARCO® ICP-OES	ELAN DRC-e ICP-MS
Nebulizer gas flow (mL/min)	1.00	0.89
Auxiliary gas flow (L/min)	1.00	1.02
Plasma gas flow (L/min)	12	15.00
RF power (W)	1400	1150
Lens voltage (V)	9.75	6.75
Peristaltic pump rate (rpm)	1.60	2.00

2.3.3. Analysis of a Certified Reference Material

The method was validated using certified reference materials (CRMs) soil, and Chinese bush branches and leaves (NCS DC 73348, National Analysis for Iron & Steel, Beijing, China; SRM 1944, New York Waterway Sediments, National Institute of Standards and Technology, New York, NY, USA). The CRMs were digested and analyzed similarly to the soil, leaves and roots of *L. perenne*.

2.4. Enzyme Extraction and Assay

To investigate the mechanism(s) used by *L. perenne* to adapt and tolerate abiotic stress, the following stress enzymes were studied: amylases, peroxidases, superoxide dismutases and glutathione transferases.

2.4.1. Total Amylolytic Activity (TAA)

The enzyme was extracted following the method by Mokgalaka-Matlala et al. [21] and assayed following the colourimetric method of Fuwa [22] with slight modifications. In 2 mL Eppendorf tubes, ground freeze-dried leaves and roots weighing about 0.1 g each were placed in triplicate. To the tubes, 1 mL of 2 mM imidazole buffer (pH 7) from Sigma Aldrich (Johannesburg, South Africa) was then added. The samples were shaken for 20 min at 200 rpm on a Labcon platform shaker and then centrifuged for 20 min at 25 °C at 21952 RCF. After transferring the supernatant into a clean Eppendorf tube, the extraction was carried out twice more. A 1% starch solution was freshly prepared by dissolving water-soluble starch in 2 mM imidazole buffer (pH 7). A total of 400 µL of the extract and a 700 µL aliquot of this solution were put into a 2 mL Eppendorf tube and thoroughly mixed. A 150 µL aliquot of the starch/extract mixture was transferred into 200 µL of cold trichloroacetic acid (Sigma Aldrich, Johannesburg, South Africa) to stop the reaction at different time intervals (0, 20, 40, 60 and 80 min). Iodine reagent (Sigma Aldrich) was prepared (0.0075% iodine in 0.075% KI), and 300 µL was transferred into the wells of a 96-well microplate. A 30 µL aliquot of the stopped reaction mixture was then transferred into wells containing iodine, in triplicate. The microplate was incubated for 20 min at room temperature, after which the absorbance was measured at 660 nm every 20 min using the Spectramax 190 (Molecular Devices, Sunnyvale, CA, USA) microplate reader. A calibration curve was prepared using starch and iodine reagent.

2.4.2. Glutathione S-Transferase Activity (GST)

The glutathione S-transferase enzyme was extracted and assayed as per the method of Melato et al. [23] with slight modification. Ground, freeze-dried leaves and roots weighing about 0.1 g each were placed in three 2 mL Eppendorf tubes with 1 mL of 0.1 M imidazole buffer (pH 6.5) added. The mixture was then shaken at 200 rpm for 20 min and centrifuged at 1792 RCF at 25 °C for 20 min. The extraction was carried out once more after the supernatant was transferred into a clean Eppendorf tube. A reaction solution was made by combining 100 µL of 112.5 mM L-glutathione (Sigma Aldrich, Johannesburg, South Africa) with 100 µL of 112.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Aldrich, Johannesburg, South Africa). The resulting mixture was then transferred into the wells of a 96-well microplate. The extract was diluted by mixing 60 µL of the extract with 140 µL of 0.1 M imidazole buffer (pH 6.5), which was then added to the microplate wells containing the reaction solution. The absorbance was then measured using the Spectramax microplate reader at 340 nm once every minute for 6 min at 25 °C, with 5 s of shaking before each reading.

2.4.3. Peroxidase Activity (POD)

The peroxidase activity was determined following the method reported by Melato et al. [23]. The following reagents were added to the 2 mL Eppendorf tubes: 0.1 g of crushed, freeze-dried leaves and roots, 0.001 g of bovine serum albumin (Merck) and 0.001 g of polyvinyl pyrrolidone (Sigma Aldrich). The mixture was shaken for 20 min and then

centrifuged at 21952 RCF for 15 min. After being transferred to a clean Eppendorf tube containing 0.001 g of Dowex Resin (Sigma Aldrich, Johannesburg, South Africa), the extract was shaken for 20 min and then centrifuged for 10 min at 21952 RCF. To a 15 mL centrifuge tube containing a 50 µL aliquot of the sample extract was then added 2 mL phosphate buffer, 30 µL H_2O_2 and 50 µL of 18.2 mM guaiacol (Sigma Aldrich, Johannesburg, South Africa). The sample was then transferred to a 96-well microplate, and a Spectramax microplate reader was used to measure the absorbance at 430 nm for 5 min. The absorbance change was recorded every 30 s.

2.4.4. Superoxide Dismutase Activity (SOD)

In triplicate, 0.1 g of ground, freeze-dried leaves and roots were measured into 2 mL Eppendorf tubes. After the addition of 1 mL of 0.067 M phosphate buffer (pH 7.6), the mixture was shaken on a platform shaker for 20 min at 200 rpm. At 25 °C and 21952 RCF, the mixture was centrifuged for 15 min. After, the supernatant was transferred into a clean Eppendorf tube. A 1.2 mL aliquot of the phosphate buffer (0.067 M, pH 7.6) was transferred into a 15 mL centrifuge tube, followed by 0.1 mL of 186 µM phenazinemethosulfate (Sigma Aldrich), 0.3 mL of 300 µM nitrobluetetrazolium (Sigma Aldrich), 0.2 mL sample extract and, finally, 1 mL deionised water. The mixture was then transferred to a water bath, set to 30 °C and the reaction was initiated by adding 0.2 mL of 780 µM nicotinamide adenine dinucleotide (NADH) (Sigma Aldrich). The mixture was incubated for 90 s, and the reaction was arrested by the addition of 1 mL of glacial acetic acid. The reaction mixture was then shaken in 4 mL of n-butanol (Sigma Aldrich) and left to stand for 10 min. The stopped reaction mixture was then centrifuged at 1792 RCF for 5 min. The intensity of chromogen in the butanol layer was measured at 560 nm using the Spectramax microplate reader.

2.5. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to study stress-induced morphological changes in *L. perenne* tissues. Leaves and roots were treated with a 2.5% glutaraldehyde (GA)/formaldehyde fixative solution. The fixative solution was prepared by mixing 1 mL of 25% GA (Sigma Aldrich), 1 mL of 25% formaldehyde (Sigma Aldrich), 3 mL deionised water and 5 mL phosphate buffer (Sigma Aldrich) (1.5 M phosphate buffer, diluted 1:1 in deionised water) for 1 h. The fixative was removed and the plant samples were rinsed 3 times with phosphate buffer, leaving them in the buffer for 15 min with each wash. After washing, the samples were dehydrated by washing with a graded series of ethanol (30%, 50%, 70% and 90%), leaving the samples in each concentration for 15 min. Finally, the samples were washed four times with 100% alcohol, leaving them in the ethanol for 15 min with each of the first three washes and then 30 min for the last wash. This was followed by a soak in a (50:50) hexamethyldisilazane (HDMS)/ethanol solution for 1 h in a covered container, under a fume hood. The HMDS/ethanol solution was then removed, and fresh HMDS was added to the samples and left open to dry overnight. The samples were then mounted on aluminium stubs and coated with carbon before being examined under the scanning electron microscope (Zeiss Crossbeam 540 FEG-SEM, Oberkochen, Germany).

2.6. Statistical Data Analysis

The results of this study are presented as the mean ± standard deviation. All statistical analyses conducted on the data were computed using Microsoft Excel software 2016. Analysis of variance (ANOVA) was used to determine significant differences in the effect of metal-induced stress on the growth, metal accumulation, TAA, GST, POD and SOD in *L. perenne* at a $p < 0.05$ significance level.

3. Results and Discussion

3.1. pH and Electrical Conductivity of the Soil

The pH and electrical conductivity are presented as the mean ± standard deviation. The landfill soil had a significantly higher pH than the control soil over the entire 18-week

pot-trial period. However, both types of soil seemed to maintain their pH values, without any significant changes throughout the experiment. The landfill soil maintained a pH between 7.7 and 8.1, while the control soil ranged between 4.8 and 5.6 (Figure 1). Elbehiry et al. [24] reported similar pH values for two landfill sites that they assessed and found to be alkaline with pH values ranging between 7.59 and 8.09. Soils with high alkalinity immobilise metals and reduce the risk of contaminating the surrounding environment [25]. Soil with a pH below 7 usually allows the migration of metals into leachate, as has been reported with Cu, Mn and Zn [26]. Furthermore, Adamczyk-Szabela et al. [21] reported that raising the soil pH to 10 led to an increase in the uptake of Cu and Mn.

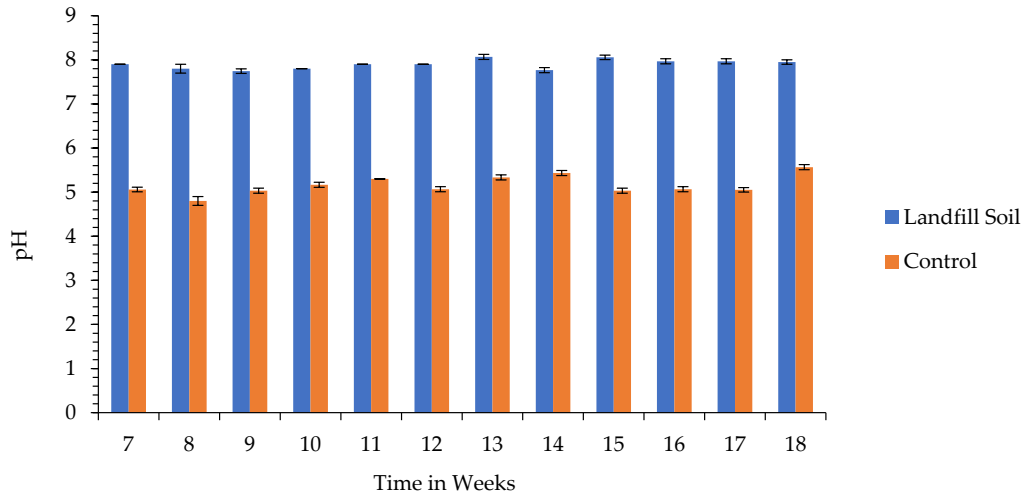


Figure 1. pH of control soil and landfill soil over the 18-week pot trial.

Electrical conductivity (EC), on the other hand, followed a completely different trend as depicted in Figure 2. Electrical conductivity values decreased gradually over the experimental period and declined to almost a quarter of the initial value for the landfill soil by the end of the trial at week 18. Control plants also displayed a decrease in conductivity, although to a lesser extent. The reduction in electrical conductivity could be attributed to reduced metal ions in the soil as a result of the assimilation of metal ions by the plants. In a previous study [23], vetiver grass grown on gold mine tailings exhibited the ability to significantly take up metals from the soil in which it was growing. This led to a decrease in the metal content of the soil with a corresponding decrease in the soil's electrical conductivity. Electrical conductivity is a measure of metal ions in the soil. This parameter gives an indication of the nutrient availability and soil health, among other things [27].

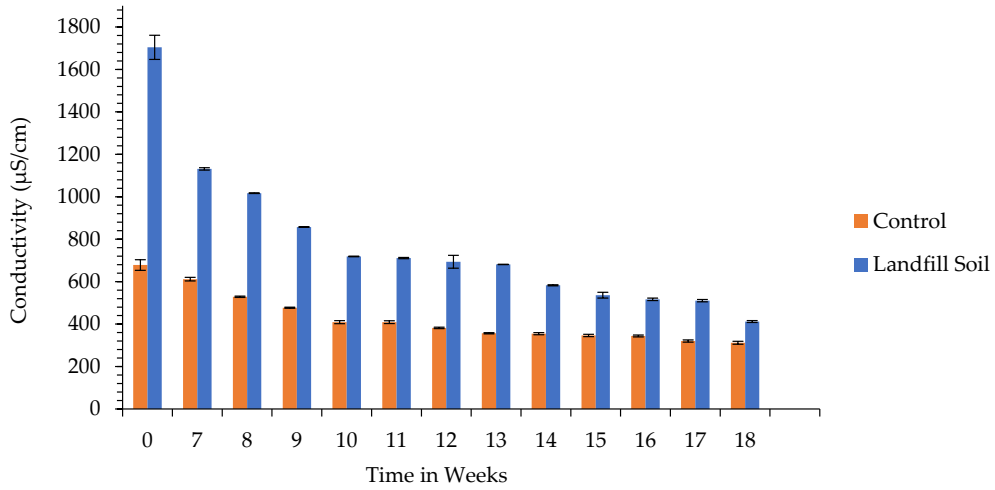


Figure 2. Electrical conductivity for control soil and landfill soil over the 18-week pot trial.

3.2. Plant Growth and Metal Uptake

L. perenne displayed the ability to grow and thrive in metal-contaminated landfill soil. In contrast, the roots and leaves of control plants were thinner and shorter than plants grown in landfill soil (Figure 3).

Figure 3. Comparison of growth of *L. perenne* plants (**A**) growing in landfill soil to (**B**) plants growing in control soil.

The ability of *L. perenne* to tolerate oxidative stress is indicated by the lengthening of the roots and leaves of the plants grown in contaminated landfill soil. Any uptake of metals from the soil into the plant initially comes into contact with the roots. Plants localize the metals to the roots and limit translocation to the leaves and, thus, avoid the induction of additional stress. As a defense mechanism against metal toxicity, plants have the ability to monitor and regulate the movement of metals from the soil through the roots to other organs [9]. Should toxic metals make it through this line of defense, the plant needs to employ tolerance mechanisms [28], one way that plants achieve this is by increasing antioxidant enzyme activity as a detoxification mechanism [29].

Validation of the Analytical Method

The method used in the analysis of the concentrations of metals in the soil and plant organs was validated using certified reference materials (CRM). The bush branches and leaves (NCS DC 73348) CRM was found to be the most appropriate for validation of the results for the leaves and roots obtained using both ICP-OES and ICP-MS. The New York Waterway sediment (SRM 1944) was used to validate the soil results. The Student *t*-test revealed no significant difference between the measured and the certified values for both CRMs at the 95% confidence level.

3.3. Metal Uptake from Landfill Soil

One major difference noted between the *L. perenne* control plant and plants growing in landfill soil, other than the physical appearance of the plants, was the Mn accumulation in

plant biomass. The uptake of Mn was significantly higher in control plants (97.6 mg/100 g total uptake in the roots and 65.2 mg/100 g uptake in the leaves) than in plants growing in landfill soil (57.9 mg/100 g in the roots and 46.4 mg/100 g in the leaves). It has been reported that in acidic conditions, the concentration of Mn^{2+} in soil solution increases, resulting in a higher potential for the soil to be toxic to plants grown in it [30]. The lower pH (Figure 1) of the control soil (5.16) compared to the landfill soil (7.90) correlates well with the uptake of Mn observed in this study. Excess Mn as a result of low soil pH was proven to cause growth reduction in ryegrass and clover [31]. Stunted growth and altered metabolic and physiological pathways in plants are reported to be biochemical symptoms of metal toxicity in plants [16].

The metal concentrations in the soil along with their gradual depletion are shown in Figure 4. Manganese had the highest initial (baseline) concentration (291 mg/100 g for control soil and 339 mg/100 g for landfill soil), followed by Zn, Pb and Cr, respectively, with Cu having the lowest initial concentration in both landfill and control soils, 25.8 mg/100 g and 15.8 mg/100 g, respectively (Table 2). The highest amount of Mn in the soil is from the Earth's crust [32]. Some anthropogenic sources of Mn in the environment are mining, mineral processing and municipal wastewater [32]. This could explain the elevated initial Mn concentrations in both the control and landfill soils. According to Wuana and Okieimen [33], Pb, Cr, As, Zn, Cd, Cu and Hg are the most prevalent heavy metals that are typically detected in landfill soil and are listed in decreasing order of abundance. This list indicates that Cu is less abundant than Pb, Cr and Zn, which is consistent with the findings of this investigation. Pb abundance was also found to be greater than Zn, Cr and Cu.

Table 2. Baseline metal concentrations for landfill and control soil.

Metal	Landfill Soil—Metal Concentration (mg/100 g)	Control Soil—Metal Concentration (mg/100 g)
Cr	75.1 ± 3.7	49.6 ± 8.2
Cu	25.8 ± 1.2	15.8 ± 1.1
Mn	339 ± 3.1	291 ± 10
Pb	168 ± 1.9	66.9 ± 0.26
Zn	294 ± 2.6	101 ± 8.4

For all *L. perenne* plants, Mn accumulated the most in leaf biomass, followed by Zn, Pb, Cr and then Cu, in decreasing order (Figure 5). Copper concentrations are kept to a minimum in plants, and when there is a need for the metal, plants are able to source it from the soil. The uptake of Cu is dependent on need, for example, when there is a lot of nitrogen in the environment, plants tend to require more Cu [34]. Chromium and Pb are not essential elements for plant survival. Plants have the ability to restrict the transfer of metals from the roots to the shoots when they are exposed to high concentrations of metals that threaten the homeostatic balance [9]. This explains the poor translocation of Cr and Pb by *L. perenne* in this study even though the plants had higher concentrations than Cu in the soil (Table 2).

All plants showed a gradual increase in metal concentration throughout the exposure period, and the overall trend in metal uptake for roots and leaves was Mn > Pb > Zn > Cr > Cu. The control soil contained a lot of organic matter, which could explain the high initial Mn content of this soil [32].

In the roots, Pb uptake increased significantly more than all the other metals in *L. perenne* plants growing on landfill soil (Figure 6). The initial Pb concentration was 30.2 ± 6.3 mg/100 g, while Mn had an initial concentration of 75.1 ± 2.9 mg/100 g. By the end of the trial, Pb had accumulated to 99.7 ± 5.1 mg/100 g, while Mn was at 133 ± 5.2 mg/100 g, increases of 230% and 77%, respectively.

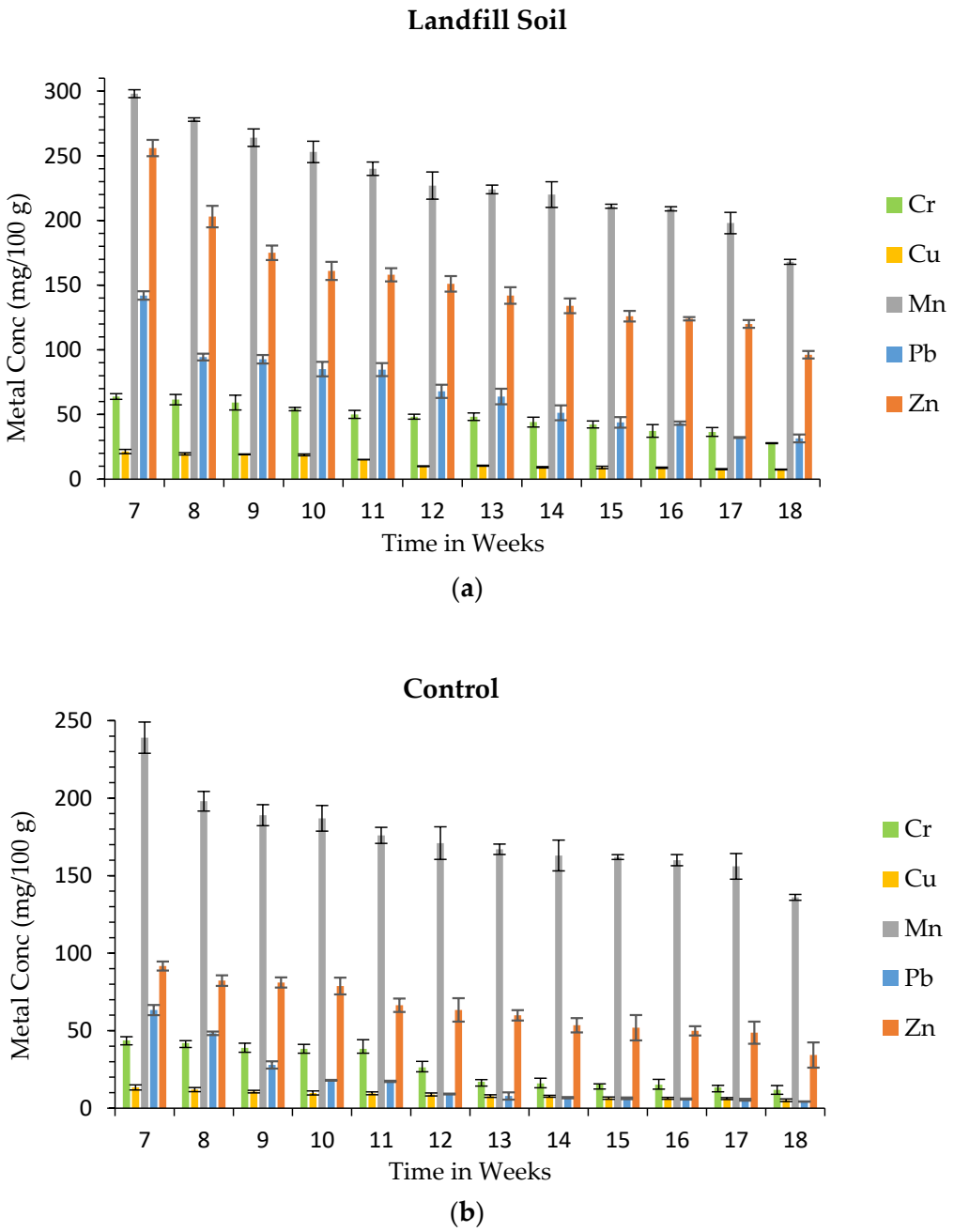


Figure 4. Metal depletion in (**a**) landfill soil and (**b**) control soil as a result of uptake by *L. perenne* plants.

In their review article, Sharma and Dubey [35] reported that Pb is able to block other metals from accessing absorption sites on the roots of plants. According to the report, Pb also has a direct impact on the concentrations of Mn in Norway spruce needles. Treatment of this plant with Pb caused a reduction in Mn and Ca concentrations.

When Chen and co-workers [36] investigated how nitric oxide affected the toxicity of cadmium in *L. perenne,* they found that the roots accumulated more Cd than the leaves. This, along with the findings in the current study confirm that *L. perenne* is capable of absorbing metals into the root matrix but might restrict further movement into other parts of the plant. This is a defense mechanism against metal toxicity. Another study corroborates this observation, stating that some plants restrict long-distance transportation of metals that could pose a threat of being toxic [9]. The overall increase in metal concentrations (Figure 6) assimilated by *L. perenne* followed the trend: Pb > Mn > Zn > Cr > Cu for the roots of plants

grown on landfill soil and Mn > Pb > Zn > Cr > Cu in the control roots. A recent study that investigated the changes in the rhizosphere biome of *L. perenne* during phytoremediation also found that this grass was able to take up Hg and Cd from contaminated soil [34]. Several other studies recently corroborated the effectiveness of this plant in the remediation of metal contaminated soil [37–39].

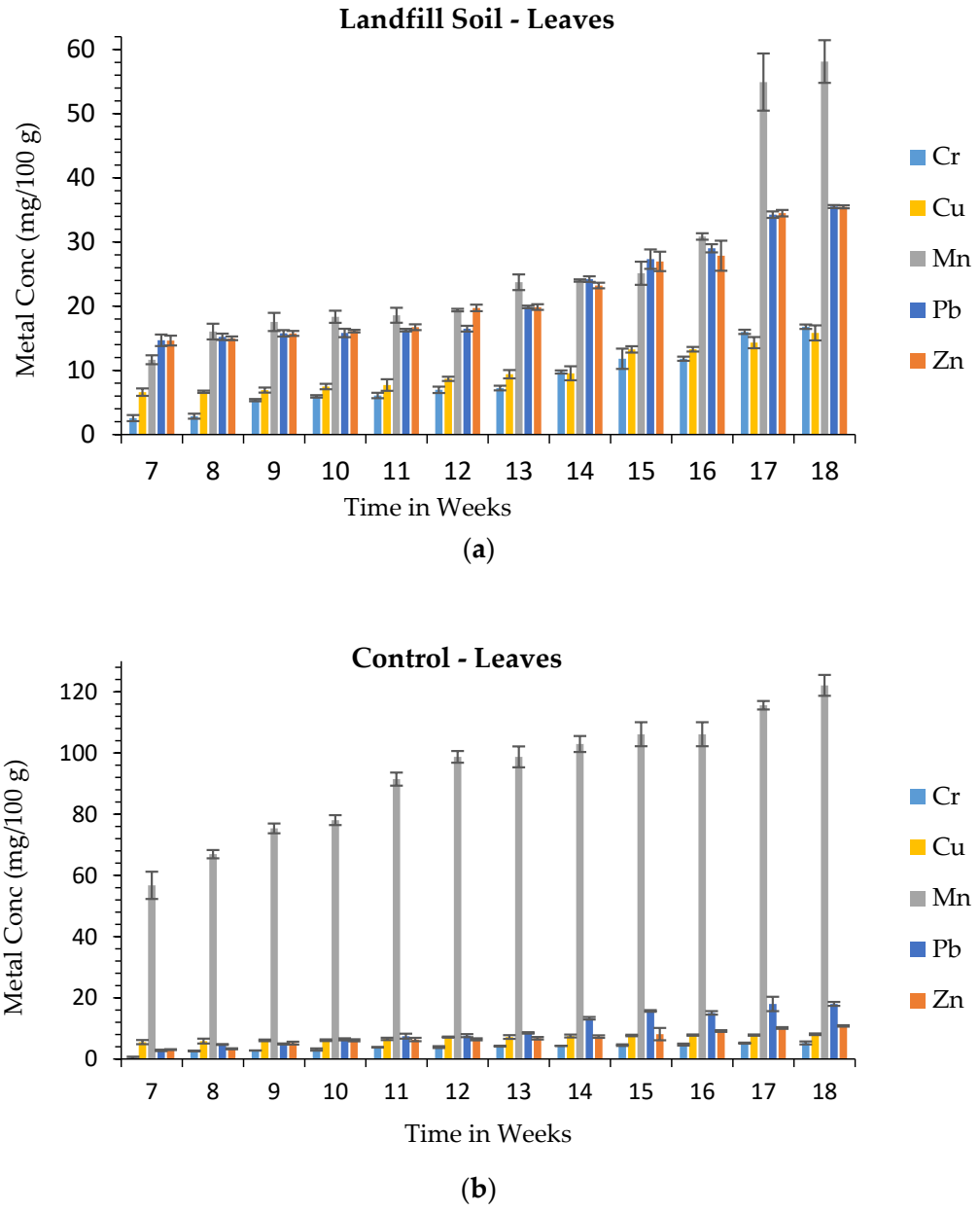


Figure 5. Metal accumulation in leaves of *L. perenne* plants grown in (**a**) landfill soil and (**b**) control soil.

3.4. Phytoremediation Potential of L. perenne

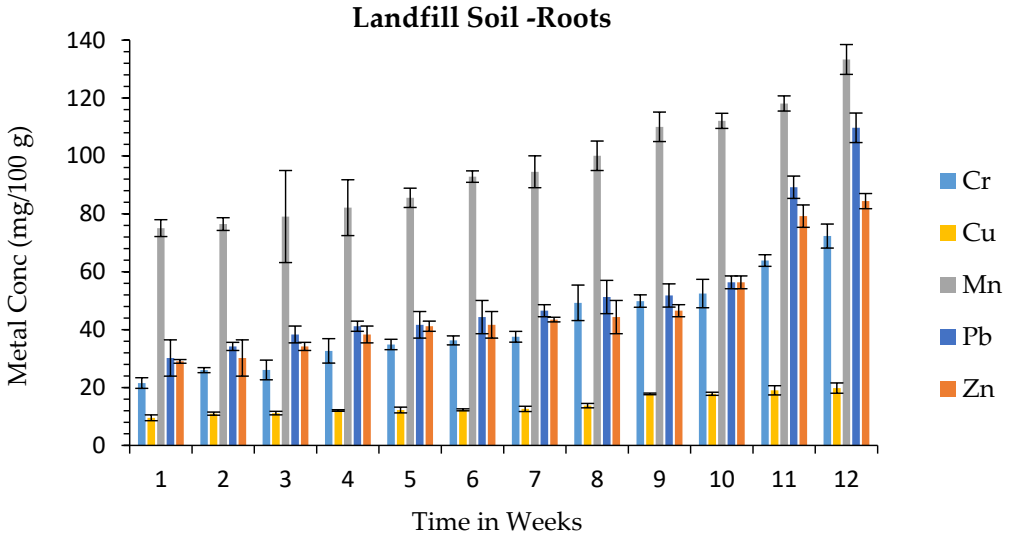
The bioconcentration factor (BCF) and translocation factor (TF) were used to estimate *L. perenne*'s potential for phytoremediation. The bioconcentration factor is a ratio of the metal concentration in the roots to the concentration in soil.

$$\text{BCF} = \frac{\text{Metal Concentration in Shoot}}{\text{Metal Concentration in Soil}}$$

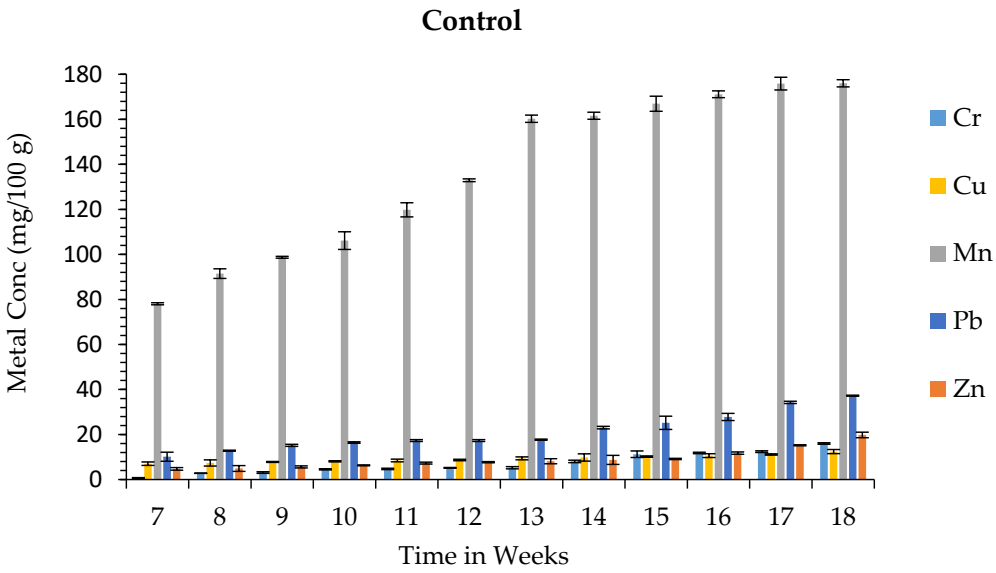
The translocation factor is the ratio of the metal concentration in the shoots to that in the roots [40].

$$\text{TF} = \frac{\text{Metal Concentration in Shoot}}{\text{Metal Concentration in Roots}}$$

A plant with a BCF and TF value > 1 is considered a bioaccumulator, while a plant with a BCF > 1 and TF < 1 can serve as a phytostabilizer. When the BCF < 1 and TF > 1, the plant could be useful as a phytoextractor [41]. The BCF and TF of *L. perenne* plants, after the 18-week growth in landfill soil, are provided in Table 3.



(**a**)



(**b**)

Figure 6. Metal accumulation in roots of *L. perenne* plants grown in (**a**) landfill soil and (**b**) control soil.

The highest BCF value for *L. perenne* grown on landfill soil was observed for Cu (1.67), followed by Cr (1.41) and then Pb (1.03). The TF of *L. perenne* was <1 for all metals studied. The low TF values (TF < 1) indicate the ability of *L. perenne* to restrict the internal movement of metals that could potentially cause great harm to the plant. This trend was coherent with the observation by Radziemska [40], where *L. perenne* accumulated significant amounts of metals in the roots than in the leaves. *Lolium perenne* has the potential to be used as a

phytostabilizer for Cu, Cr and Pb in landfill soil. Waterlot and Hechelski [42] also explored a similar study and found that *L. perenne* had the potential to serve as a phytostabilizer in the presence of amendments in the form of calcium dihydrogen phosphate and calcium hydrogen phosphate.

Table 3. BCF and TF of *L. perenne* growing on landfill soil.

Metal	BCF		TF	
	Plants Grown on Landfill Oil	Control Plants	Plants Grown on Landfill Soil	Control Plants
Cr	1.41	0.49	0.25	0.33
Cu	1.67	1.53	0.80	0.65
Mn	0.64	1.25	0.43	0.69
Pb	1.03	0.87	0.32	0.48
Zn	0.47	0.33	0.42	0.55

3.5. Scanning Electron Microscopy

The structural and morphological changes in the *L. perenne* plant tissues that might have been caused by stress factors, such as metals and pH, among others, were examined using scanning electron microscopy (SEM). Scanning electron micrographs show the epidermis on the top surface of the plants grown on landfill soil (Figure 7a), which appears thicker than that of the control plant (Figure 7b). Fan and Neumann [43] reported that an increase in lignin metabolism by stress factors such as water deficit in maize inhibited cell extensibility which, in turn, led to the stiffening of the cell wall. Furthermore, plants thicken their cell wall to decrease their permeability and, thus, prevent the migration of toxic metals into sensitive internal parts of the plant such as the protoplasm [44].

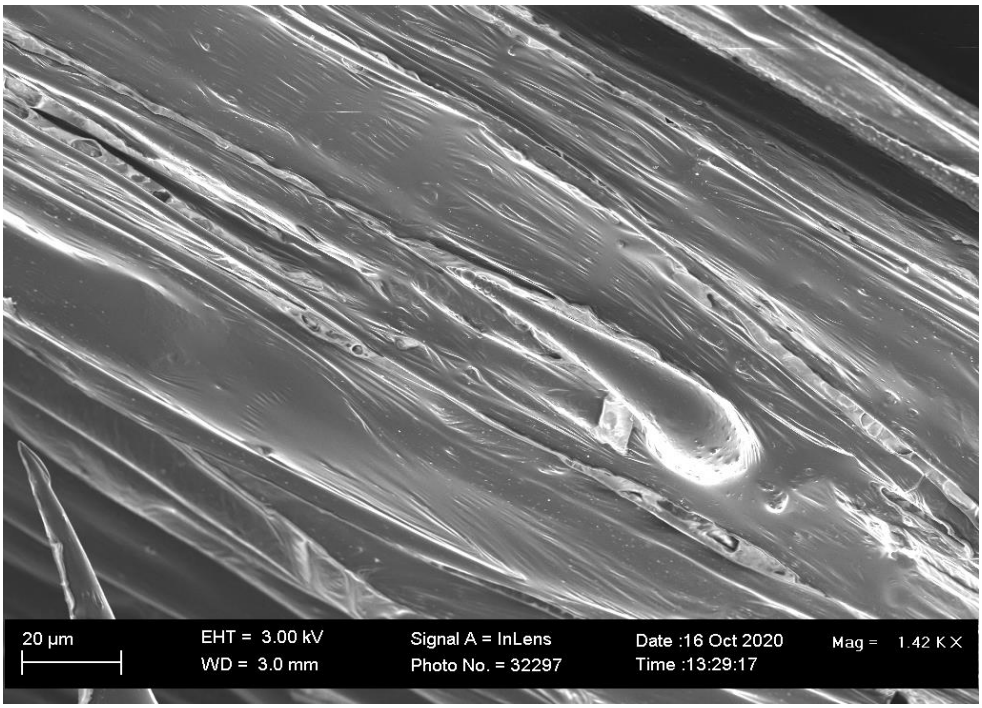
In comparison to control leaves, the leaves of plants grown in landfill soil had larger and more pronounced trichomes on their surface (Figure 7a). Trichomes can serve as a storage site for metals, where detoxification can take place. They can also assist the plant by secreting secondary metabolites that can mitigate the hazardous effects of metals [45].

The SEM micrograph of the bottom side of the plants grown on landfill soil (Figure 8a) showed an increased cuticular wax layer compared to the control plants (Figure 8b). Plants are shielded from external stress by the waterproof cuticular wax layer. The waxy layer has also reportedly been shown to reduce residual transpiration, lessen nonstomatal water loss, shield plants from UV radiation and minimize the retention of dust and air pollutants [46]. According to Seo and Park [47], cuticular wax deposition correlates with plant responses to cellular dehydration. Tobacco (*Nicotiana glauca* L. *Graham*) leaves grown under periodic dehydration stress had a more cuticular wax load in a study by Cameron et al. [48].

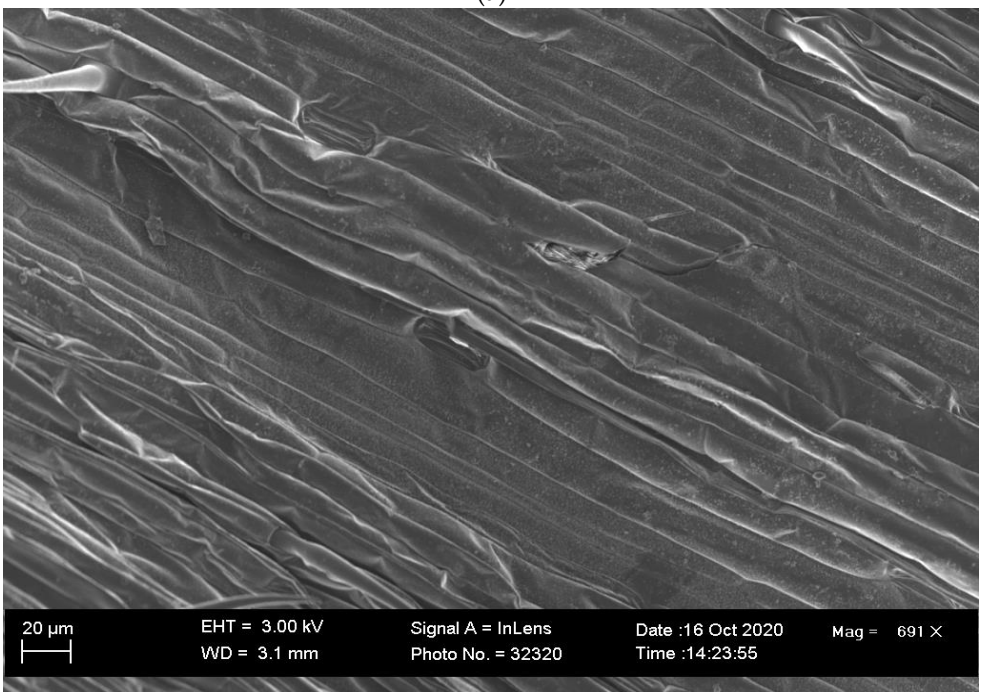
3.6. Enzyme Activity

3.6.1. Superoxide Dismutase Activity

All cells that metabolise oxygen have superoxide dismutase (SOD) enzymes in their mitochondria, nucleus, and peroxisomes, among other cellular compartments [49]. These enzymes form part of the first line of defense against increasing reactive oxygen species (ROS) concentrations and are also considered to be central to antioxidant defense mechanisms in plants [50]. Superoxide dismutase activity increased with increasing exposure time and was found to be significantly higher ($p < 0.05$) in the leaves than in the roots (Figure 9). Plants grown in landfill soil showed twice the amount of SOD activity in their leaves compared to control plants' roots. The SOD activity was 0.72 ± 0.02 U in the leaves and 0.51 ± 0.02 U in the roots of plants grown on landfill soil at the end of the trial. Other studies have also found similar results with regard to the SOD activity being lower in the roots than in the leaves [36,51]. Superoxide and peroxidase were reported to also show a positive response to increased Mn concentrations in the plant tissue of Macleaya cordata (Willd.) R. Br. [52].



(**a**)



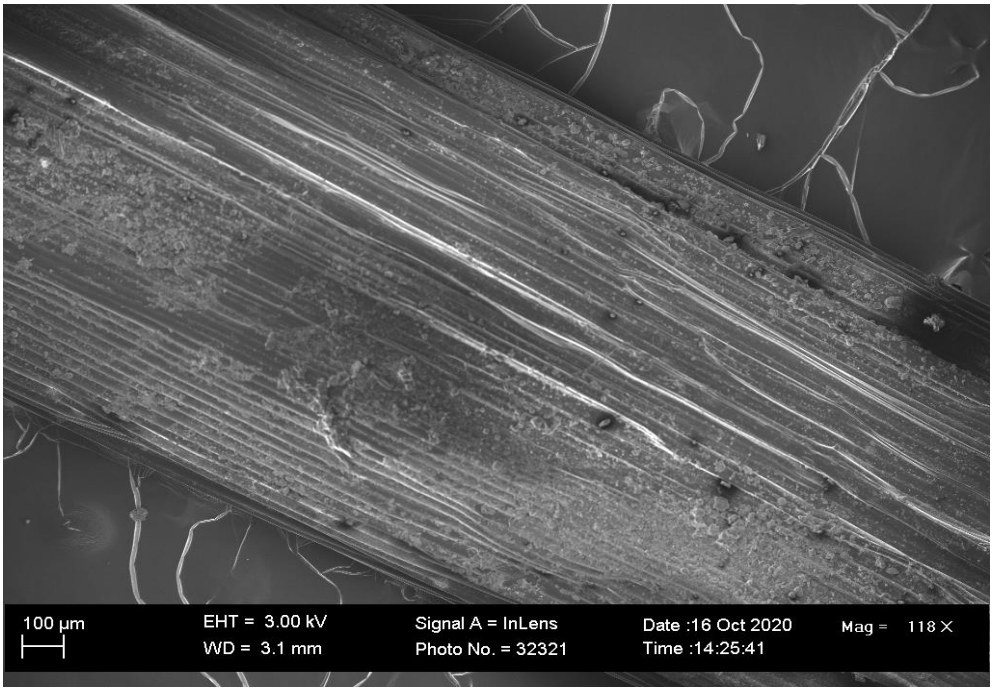
(**b**)

Figure 7. SEM micrographs showing the top surface of the leaves of *L. perenne* plants grown in (**a**) landfill soil and (**b**) the control plants.

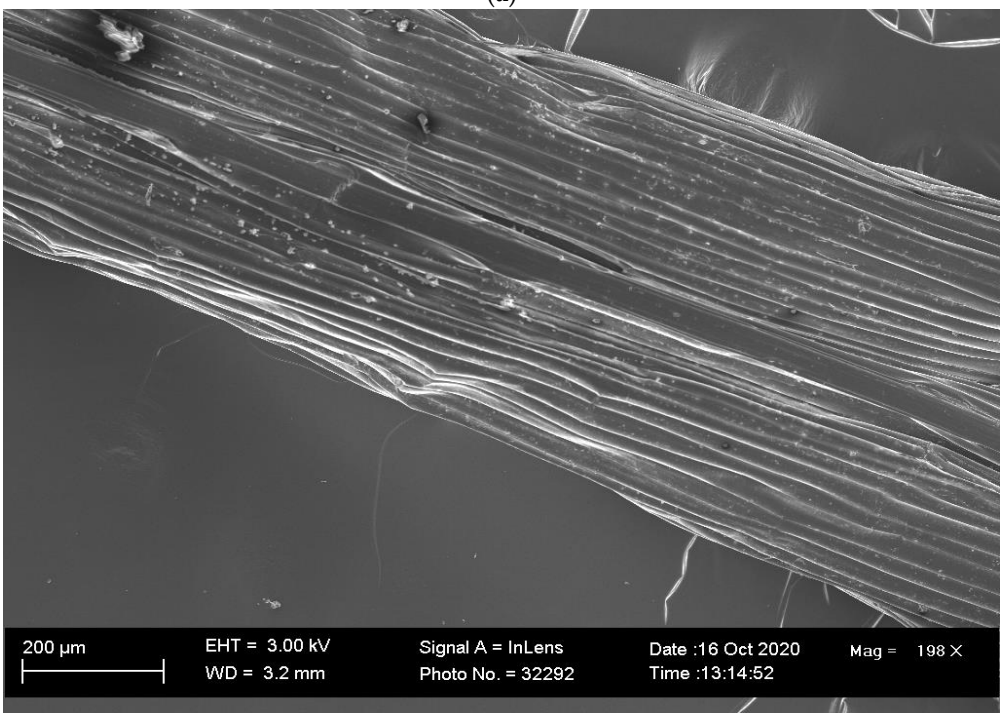
3.6.2. Glutathione Transferase Activity

In living systems, glutathione transferases catalyse the detoxification of a variety of xenobiotics by facilitating the conjugation of the glutathione's thiol group with electrophilic xenobiotic compounds. The conjugation of the glutathione thiol group with CDNB mimics the reaction between glutathione and xenobiotic compounds, with an increase in absorbance at 340 nm being an indication of the conjugation of CDNB with glutathione [53]. The glutathione S-transferase (GST) enzyme activity of *L. perenne* gradually increased in the

plants grown in landfill soil. The behaviour of the roots and leaves was similar, with the enzyme activity in the leaves being significantly greater than that of the roots (Figure 10). A study that investigated the detoxification mechanisms of *Chrysopogon zizanioides* (vetiver grass) growing in gold mine tailings reported similar findings. It was discovered that the GST activity was much higher in leaves than in roots [23].



(**a**)



(**b**)

Figure 8. SEM micrographs showing the bottom surface of the leaves of *L. perenne* plants grown in (**a**) landfill soil and (**b**) control plants.

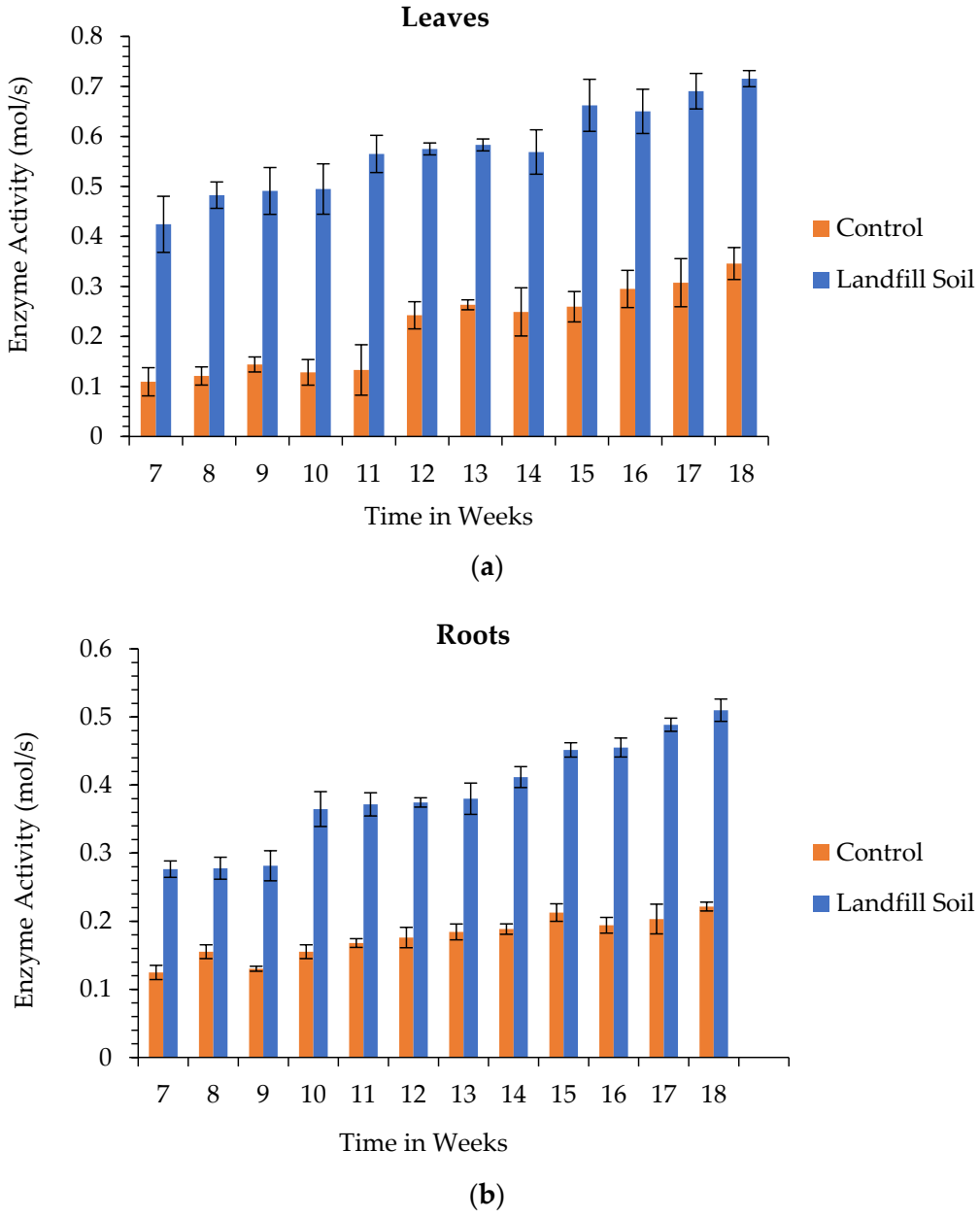


Figure 9. Superoxide dismutase activity for (**a**) leaves and (**b**) roots of *L. perenne* plants growing on landfill soil and the control soil.

3.6.3. Peroxidase Activity

Elevated peroxidase (POD) activity is an early response to stress in plants and provides cells with a form of defense against increased H_2O_2 production. There are several different kinds of peroxidases, and the activity of each kind is influenced by environmental factors such as the season of the year, the temperature, and the kind of stress to which the organism is subjected [54,55]. With increasing exposure time, the POD activity in the leaves and roots of *L. perenne* plants grown in landfill soil increased and was substantially ($p < 0.05$) higher than the POD in control plants (Figure 11). In comparison to the roots, the POD in the leaves was substantially higher. When compared to the POD in the roots, which ranged from 16.9 U to 40.8 U, the POD of the leaves of plants growing on landfill soil was approximately 1.5 times more and ranged from 19.5 U to 52.8 U. A similar response was seen in *M. cordata* when grown in the sand with increasing Mn concentrations. Peroxidase activity increased with Mn concentration [52].

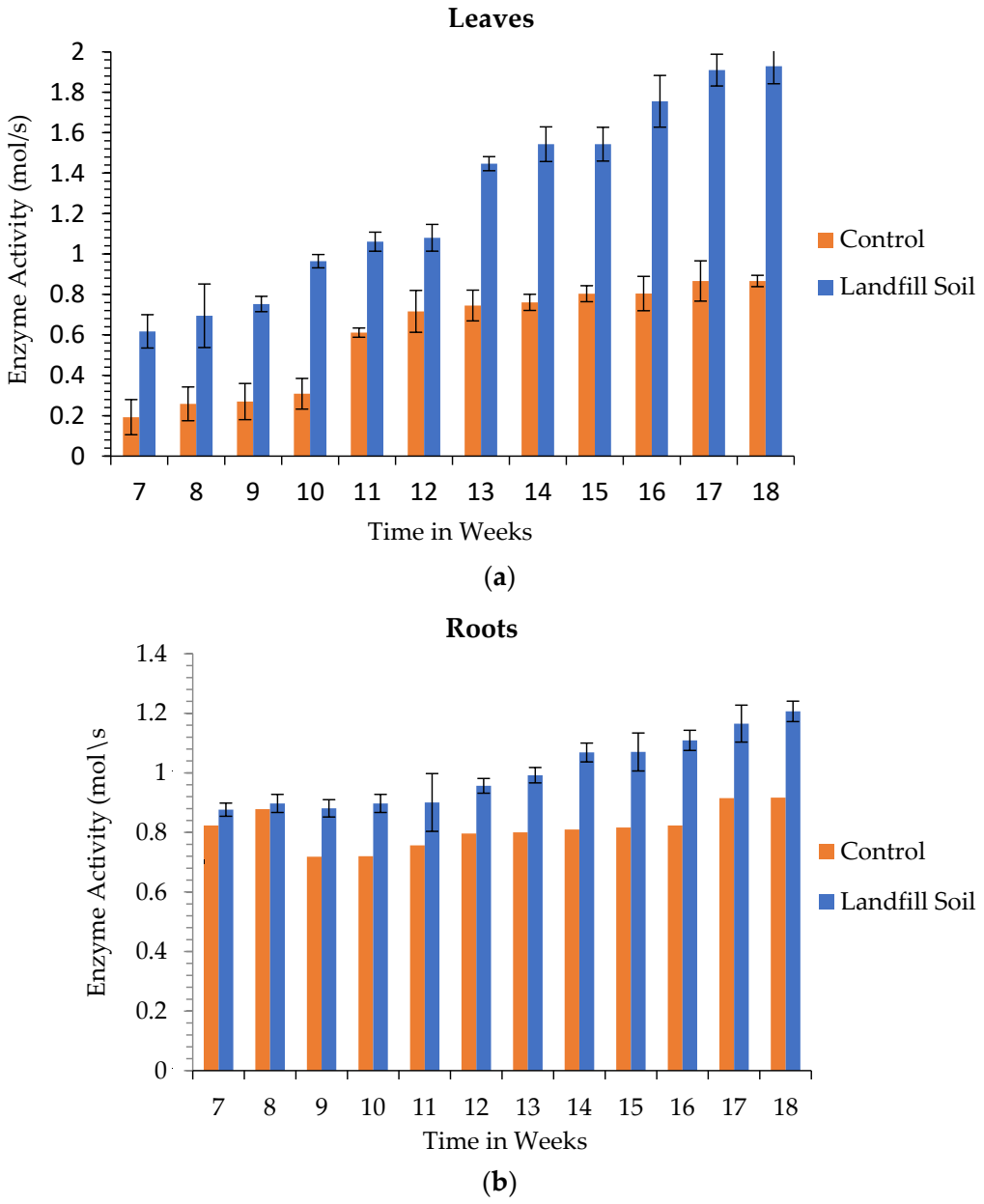


Figure 10. Glutathione transferase activity for (**a**) leaves and (**b**) roots of *L. perenne* plants growing on landfill soil and the control soil.

Anything absorbed by plants from the soil first comes into contact with the roots. Since the greatest amounts of metals were found in the roots of *L. perenne*, this could suggest that a significant amount of damage to root tissue might have resulted in lower levels of the POD enzyme activity. Another possibility, contrary to this, could be that the lower level of peroxidase activity in the roots might point to a greater tolerance of metal stress in the roots than in the leaves [56].

3.6.4. Total Amylolytic Activity

The total amylolytic activity (TAA) of the plants grown in landfill soil was found to be higher than that of the control plants (Figure 12). The leaves of *L. perenne* exhibited higher enzyme activity overall, with the TAA of leaves of plants growing in landfill soil ranging from 0.0018 U to 0.0094 U, compared to the roots (0.0014 U to 0.0051 U). The amylolytic activity for the leaves seems to almost double from week 14 to 15 and continued to maintain a high activity for the last 4 weeks of the experiment. For the roots, a major increase in TAA

was seen two weeks before the conclusion of the experiment. The low enzyme activity in the first few weeks could be due to the fact that the plants were still acclimatizing to their new environment. It has been reported that some plants have higher amylase activity in the leaves than in the roots even when growing under ambient conditions [54].

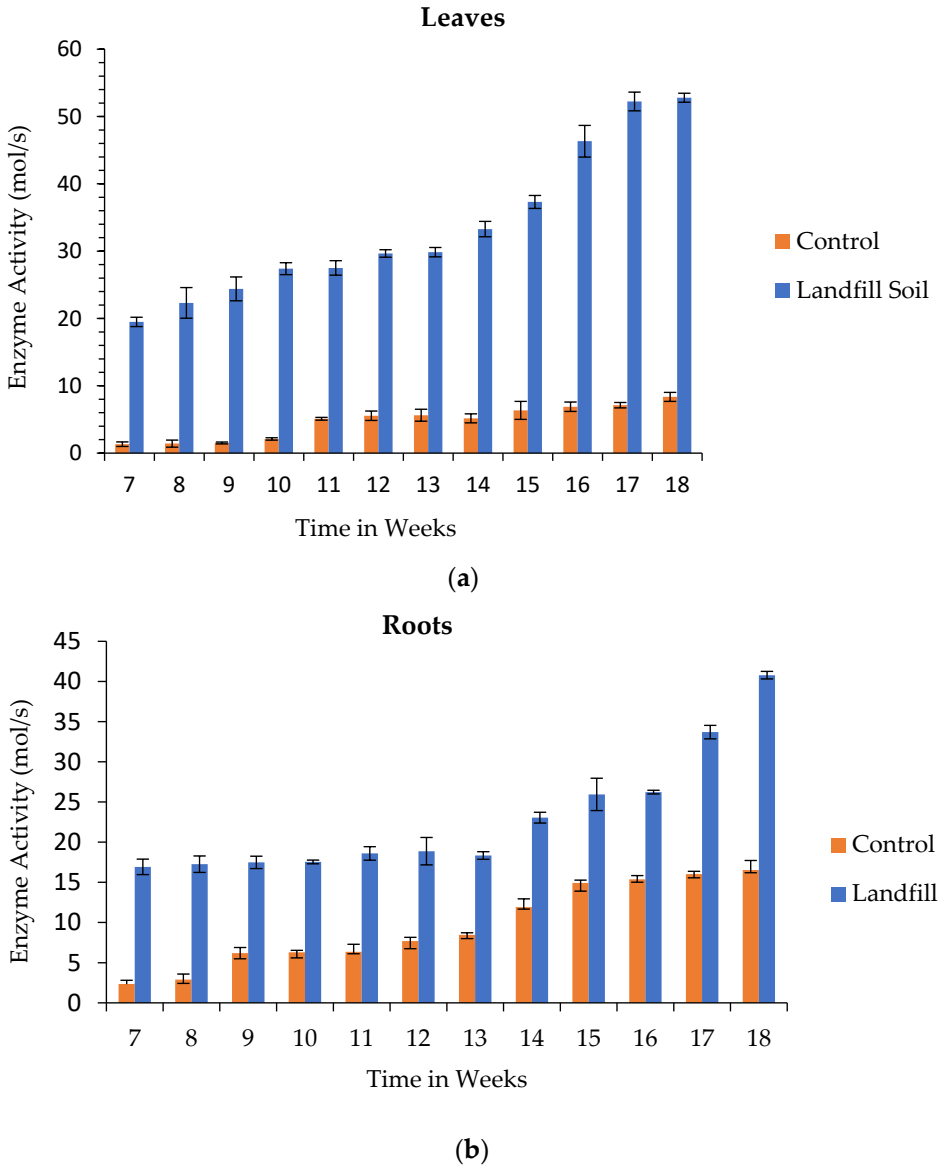


Figure 11. Peroxidase activity for (**a**) leaves and (**b**) roots of *L. perenne* plants growing on landfill soil.

Through photosynthesis, plants can harness sunlight and use it to fix carbon and produce glucose [57]. When the need arises, excess glucose can be stored as starch molecules. Amylases play a significant role in metabolising these starch molecules, allowing plants to harness the stored energy [58]. Both α and β amylases facilitate starch hydrolysis into its component sugars which plants can use to supply their energy needs. Metal toxicity has been found to disrupt several essential processes and pathways in plant metabolism [59]. Amirjani [60] found that total amylolytic activity dropped by 26.8% as Cd concentrations increased in the growth medium of germinated wheat seed. Under stressful conditions, plants increase enzyme activity to mitigate the effects of stress-causing factors [61]. Starch breakdown in response to stress is often linked to increased stress tolerance in plants. To generate energy and create sugars and other metabolites that assist to reduce stress, research has shown that plants frequently mobilise their starch reserves [62]. This suggests

that more amylase would be employed in order to cater to the energy needs of the plant under stress; this justifies the observed increase in TAA.

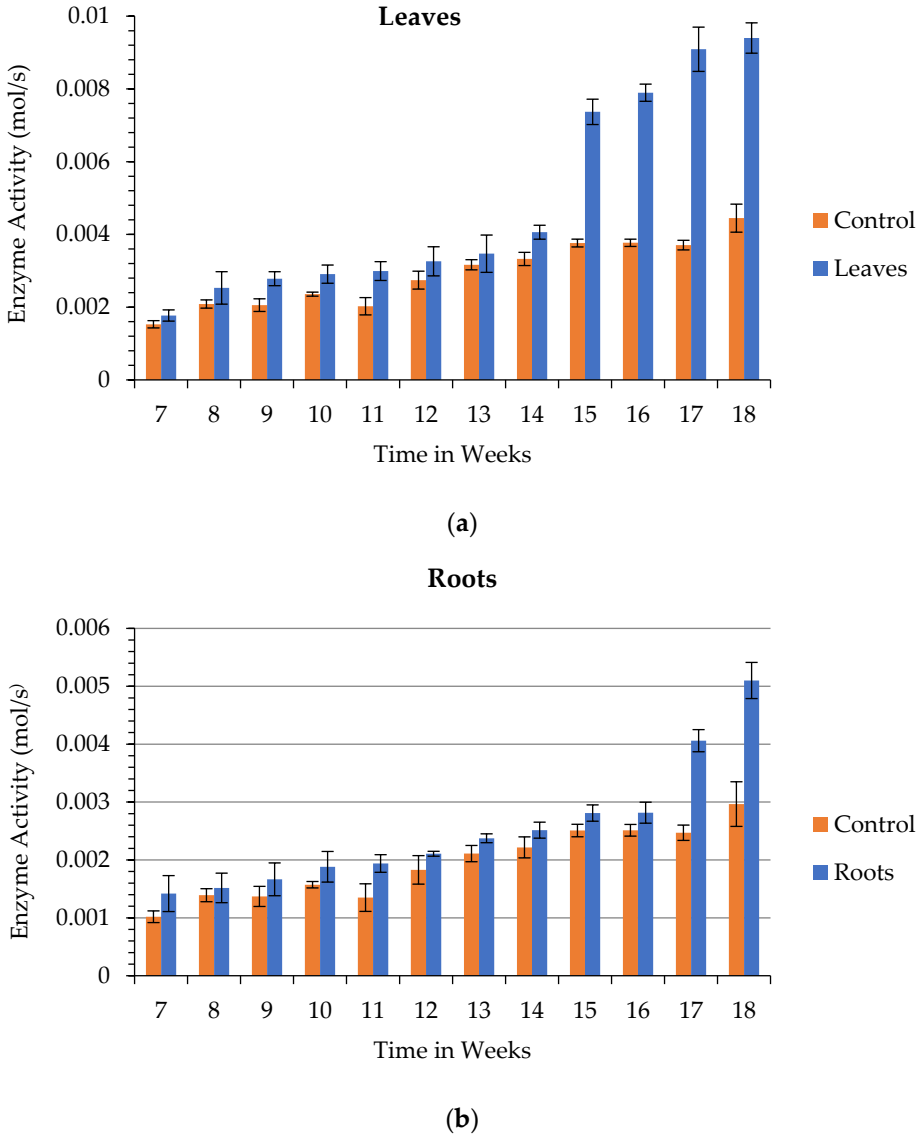


Figure 12. Total amylolytic activity for (**a**) leaves and (**b**) roots of *L. perenne* plants growing on landfill soil.

4. Conclusions

L. perenne was able to thrive for 18 weeks in metal-containing landfill soil without exhibiting any signs of phytotoxicity. The plants in landfill soil had wider leaf blades and longer, thicker and more numerous roots (Figure 3) and also appeared greener and healthier than the control plants. Infiltration by leachates is a primary pathway of migration of contaminants to groundwater; therefore, *L. perenne* roots may play an important role in regulating the water content in landfills, limiting possible groundwater pollution. The plant-accumulated metals found in the landfill soil, in its roots with corresponding translocation factor values of all the metals < 1 (values ranged between 0.2 and 0.8). On the contrary, Cu, Cr and Pb exhibited BCF values above 1, demonstrating the plant's ability to assimilate metals from the soil. The low TFs and high BCFs render *L. perenne* a potential candidate for the phytostabilization of Cu, Cr and Pb in landfill soil. In addition to the uptake of metals, the enzymes SOD, GST, POD and TAA displayed a gradual increase in activity over a 18-week exposure period, with POD showing the highest enzyme activity and TAA having

the largest increase in enzyme activity. Plants increase the production of enzymes and other antioxidants to prevent or minimise oxidative damage under stressful conditions [63]. The findings of this study confirm the ability of *L. perenne* to detoxify itself from metal-induced stress through activation and increasing antioxidant enzymes, thickening of cell walls and increased cuticular wax layer of the leaves to decrease their permeability by toxic metals, as well as enlarging the trichomes as storage and detoxification sites for metals. *L. perenne* is a potential candidate for phytoremediation. Because of its sedentary nature, over a period of 18 weeks, the plant developed mechanisms to abstract metals from landfill soil and control the geochemical conditions of the landfill soil by significantly reducing the electrical conductivity.

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References

1. Kim, K.R.; Owens, G. Potential for enhanced phytoremediation of landfills using biosolids—A review. *J. Environ. Manag.* **2010**, *91*, 791–797. [CrossRef] [PubMed]
2. The World Bank Annual Report. Available online: https://thedocs.worldbank.org/en/doc/596391540568499043-0340022018/original/worldbankannualreport2016.pdf (accessed on 28 September 2016).
3. Faiza, M.T.; Hassan, N.A.; Mohammad Farhan, R.; Edre, M.A.; Rus, R.M. Solid Waste: Its Implication for Health and Risk of Vector Borne Diseases. *JWBM* **2019**, *1*, 14–17.
4. Aljaradin, M.; Persson, K.M. Municipal Landfilling Practice and its Impact on the Water Resources-Jordan. *World Environ.* **2014**, *5*, 213–218.
5. Jayawardhana, Y.; Kumarathilaka, P.; Herath, I.; Vithanage, M. Municipal Solid Waste Biochar for Prevention of Pollution from Landfill Leachate. In *Environmental Materials and Waste*; Academic Press: Hong Kong, China, 2016; pp. 117–148.
6. Bouzayani, F.; Aydi, A.; Abichou, T. Soil Contamination by Heavy Metals in Landfills: Measurements from an Unlined Leachate Storage Basin. *Environ. Monit. Assess.* **2014**, *186*, 5033–5040. [CrossRef]
7. Wan, X.; Lei, M.; Chen, T. Cost–Benefit Calculation of Phytoremediation Technology for Heavy-Metal-Contaminated Soil. *Sci. Total Environ.* **2016**, *563*, 796–802. [CrossRef] [PubMed]
8. Purakayastha, T.J.; Viswanath, T.; Bhadraray, S.; Chhonkar, P.K.; Adhikari, P.P.; Suribabu, K. Phytoextraction of Zinc, Copper, Nickel and Lead from a Contaminated Soil by Different Species of *Brassica*. *Int. J. Phytoremediation* **2008**, *10*, 63–74. [CrossRef]
9. Ghori, Z.; Iftikhar, H.; Bhatti, M.F.; Sharma, I.; Kazi, A.G.; Ahmad, P. Phytoextraction: The Use of Plants to Remove Heavy Metals from Soil. *J. Plant Interact.* **2019**, 385–409.
10. Balafrej, H.B.; Bogusz, D.; Triqui, Z.E.A.; Guedira, A.; Bendaou, N.; Smouni, A.; Fahr, M. Zinc Hyperaccumulation in Plants: A Review. *Plants* **2020**, *9*, 562–684.
11. Zalewska, M. Response of Perennial Ryegrass (*Lolium perenne* L.) to Soil Contamination with Zinc. *J. Elem.* **2012**, *17*, 329–343. [CrossRef]
12. Prasad, M.N.V.; Freitas, H.M.O. Hyperaccumulation in plants–Biodiversity and prospecting for phytoremediation studies. *Electron. J. Biotechnol.* **2003**, *6*, 285–305. [CrossRef]
13. Casler, M.D.U.; Undersander, D.J. Identification of Temperate Pasture Grasses and Legumes. In *Horse Pasture Management*; Academic Press: London, UK, 2018; pp. 11–35.
14. Vatansever, R.O.; Filiz, E. Essential and Beneficial Trace Elements in Plants, and their Transport in Roots: A Review. *Appl. Biochem. Biotechnol.* **2017**, *181*, 464–482. [CrossRef] [PubMed]
15. Millaleo, R.; Reyes-Díaz, M.; Ivanov, A.G.; Mora, M.L.; Alberdi, M. Manganese as Essential and Toxic Element for Plants: Transport, Accumulation and Resistance Mechanisms. *J. Soil Sci. Plant Nutr.* **2010**, *10*, 470–481. [CrossRef]

16. Singh, S.; Parihar, P.; Singh, R.; Singh, V.P.; Prasad, S.M. Heavy Metal Tolerance in Plants: Role of Transcriptomics, Proteomics, Metabolomics, and Ionomics. *Front. Plant Sci.* **2016**, *6*, 1143–1149. [CrossRef] [PubMed]
17. Murray, K.; Rodwell, V.; Bender, D.; Botham, K.M.; Weil, P.A.; Kennelly, P.J. *Harper's Illustrated Biochemistry*; McGraw-Hill: New York, NY, USA, 2009; Volume 28, pp. 482–483.
18. Milkovic, L.C.G.; Cindric, M.; Mouthuy, P.A.; Zarkovic, N. Short Overview of ROS as Cell Function Regulators and their Implications in Therapy Concepts. *Cells* **2019**, *8*, 793–798. [CrossRef]
19. Verma, S.; Dubey, R.S. Lead Toxicity Induces Lipid Peroxidation and Alters the Activities of Antioxidant Enzymes in Growing Rice Plants. *Plant Sci. J.* **2003**, *164*, 645–655. [CrossRef]
20. Alscher, R.G.; Erturk, N.; Heath, L.S. Role of Superoxide Dismutases (SODs) in Controlling Oxidative Stress in Plants. *J. Exp. Bot.* **2002**, *53*, 1331–1341. [CrossRef]
21. Mokgalaka-Matlala, N.S.; Flores-Taviz, E.; Castillo-Michel, H.; Peralta-Videa, J.R.; Gardea-Torresdey, J.L. Toxicity of arsenic (III) and (V) on plant growth, element uptake, and total amylolytic activity of mesquite (*Prosopis Juliflora* × *P. Velutina*). *Int. J. Phytoremediation* **2008**, *10*, 47–60. [CrossRef]
22. Fuwa, H. A new method for micro determination of amylase activity by the use of amylose as substrate. *J. Biochem.* **1954**, *41*, 583–593. [CrossRef]
23. Melato, F.A.; Mokgalaka, N.S.; Mccrindle, R.I. Adaptation and Detoxification Mechanisms of Vetiver Grass (Chrysopogon Zizanioides) Growing on Gold Mine Tailings. *Int. J. Phytoremediation* **2016**, *18*, 509–520. [CrossRef]
24. Elbehiry, F.; Elbasiouny, H.; Ali, R.; Brevik, E.C. Enhanced Immobilization and Phytoremediation of Heavy Metals in Landfill Contaminated Soils. *Water Air Soil Pollut.* **2020**, *231*, 204. [CrossRef]
25. Ou, J.; Li, H.; Yan, Z.; Zhou, Y.; Bai, L.; Zhang, C.; Wang, X.; Chen, G. In situ Immobilisation of Toxic Metals in Soil using Maifan Stone and Illite/Smectite Clay. *Sci. Rep.* **2018**, *8*, 4618. [CrossRef] [PubMed]
26. Adamczyk-Szabela, D.; Markiewicz, J.; Wolf, W.M. Heavy Metal Uptake by Herbs. IV. Influence of Soil pH on the Content of Heavy Metals in *Valeriana officinalis* L. *Water Air Soil Pollut.* **2015**, *226*, 106. [CrossRef] [PubMed]
27. Corwin, D.L.; Yemoto, K. Measurement of Soil Salinity: Electrical Conductivity and Total Dissolved Solids. *Soil Sci. Soc. Am. J.* **2019**, *83*, 1–2. [CrossRef]
28. Mehes-Smith, M.; Nkongolo, K.; Cholewa, E. Coping Mechanisms of Plants to Metal Contaminated Soil. *J. Environ. Sustain.* **2013**, *54*, 53–90.
29. Hossain, M.A.; Piyatida, P.; Da Silva, J.A.T.; Fujita, M. Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. *J. Bot.* **2012**, *2012*, 872875. [CrossRef]
30. Inostroza-Blancheteau, C.; Reyes-Díaz, M.; Berríos, G.; Rodrigues-Salvador, A.; Nunes-Nesi, A.; Deppe, M.; Demanet, R.; Rengel, Z.; Alberdi, M. Physiological and Biochemical Responses to Manganese Toxicity in Ryegrass (*Lolium Perenne* L.) Genotypes. *Plant Physiol. Biochem.* **2017**, *123*, 89–97. [CrossRef]
31. Rosas, A.; Rengel, Z.; De La Luz Mora, M. Manganese Supply and pH Influence Growth, Carboxylate Exudation and Peroxidase Activity of Ryegrass and White Clover. *J. Plant Nutr.* **2007**, *30*, 253–270. [CrossRef]
32. Rollin, H.B.; Nogueira, C.M.C.A. Manganese: Environmental Pollution and Health Effects. In *Encyclopedia of Environmental Health*, 2nd ed.; Elsevier Science: San Diego, CA, USA, 2011.
33. Wuana, R.A.; Okieimen, F.E. Heavy Metals in Contaminated Soils: A Review of Sources, Chemistry, Risks and Best Available Strategies for Remediation. *ISRN Ecol.* **2011**, *2011*, 402647. [CrossRef]
34. Yruela, I. Copper in plants: Acquisition, transport and interactions. *Funct. Plant Biol.* **2009**, *36*, 409–430. [CrossRef]
35. Sharma, P.; Dubey, R.S. Lead Toxicity in Plants. *Braz. J. Plant Physiol.* **2005**, *17*, 35–52. [CrossRef]
36. Chen, W.; Dong, Y.; Hu, G.; Bai, X. Effects of Exogenous Nitric Oxide on Cadmium Toxicity and Antioxidative System in Perennial Ryegrass. *J. Soil Sci. Plant Nutr.* **2018**, *18*, 129–143. [CrossRef]
37. Leudo, A.M.; Cruz, Y.; Montoya-Ruiz, C.; Delgado, M.D.P.; Saldarriaga, J.F. Mercury phytoremediation with *Lolium perenne*-Mycorrhizae in contaminated soils. *Sustainability* **2020**, *12*, 3795. [CrossRef]
38. Rong, L.; Zhang, S.; Wang, J.; Li, S.; Xie, S.; Wang, G. Phytoremediation of uranium-contaminated soil by perennial ryegrass (*Lolium perenne* L.) enhanced with citric acid application. *Environ. Sci. Pollut. Res.* **2022**, *29*, 33002–33012. [CrossRef] [PubMed]
39. Saldarriaga, J.F.; López, J.E.; Díaz-García, L.; Montoya-Ruiz, C. Hanges in *Lolium perenne* L. rhizosphere microbiome during phytoremediation of Cd-and Hg-contaminated soils. *Environ. Sci. Pollut. Res.* **2023**, *30*, 49498–49511. [CrossRef]
40. Radziemska, M.; Bilgin, A.; Vaverková, M. Application of Mineral-Based Amendments for Enhancing Phytostabilization in *Lolium perenne* L. Cultivation. *CLEAN–Soil Air Water* **2018**, *46*, 1600679. [CrossRef]
41. Takarina, N.; Tjiong, G.P. Bioconcentration Factor (BCF) and Translocation Factor (TF) of Heavy Metals in Mangrove Trees of Blanakan Fish Farm. *Makara J. Sci.* **2017**, *21*, 6–20. [CrossRef]
42. Waterlot, C.; Hechelski, M. Benefits of Ryegrass on Multi-Contaminated Soils Part 1: Effects of Fertilizers on Bioavailability and Accumulation of Metals. *Sustainability* **2019**, *11*, 5093. [CrossRef]
43. Fan, L.; Neumann, P.M. The spatially variable inhibition by water deficit of maize root growt correlates with altered profiles of proton flux and cell wall pH. *Plant Physiol.* **2004**, *135*, 2291–2300. [CrossRef] [PubMed]
44. Krzeslowska, M. The Cell Wall in Plant Cell Response to Trace Metals: Polysaccharide Remodeling and Its Role in Defense Strategy. *Acta Physiol. Plant* **2011**, *33*, 35–51. [CrossRef]

45. Emamverdian, A.; Ding, Y.; Mokhberdoran, F.; Xie, Y. Heavy Metal Stress and Some Mechanisms of Plant Defense Response. *Sci. World J.* **2015**, *2015*, 756120. [CrossRef]
46. Ahmad, H.M.; Mahmood-Ur-Rahman, A.Q.; Awan, S.I. Plant Cuticular Waxes: A Review on Functions, Composition, Biosyntheses Mechanism and Transportation. *Life Sci.* **2015**, *2*, 60–67.
47. Seo, P.J.; Park, C.P. Cuticular Wax Biosynthesis as a Way of Inducing Drought Resistance. *Plant Signal. Behav.* **2011**, *6*, 1043–1045. [CrossRef] [PubMed]
48. Cameron, K.D.; Teece, M.A.; Smart, L.B. Increased Accumulation of Cuticular Wax and Expression of Lipid Transfer Protein in Response to Periodic Drying Events in Leaves of Tree Tobacco. *Plant Physiol.* **2006**, *140*, 176–183. [CrossRef] [PubMed]
49. Berwal, M.K.; Ram, C. Superoxide Dismutase: A Stable Biochemical Marker for Abiotic Stress Tolerance in Higher Plants. In *Abiotic and Biotic Stress in Plants*; IntechOpen: London, UK, 2018; pp. 1–4.
50. Saibi, W.; Brini, F. Superoxide Dismutase (SOD) and Abiotic Stress Tolerance in Plants: An Overview. In *Superoxide Dismutase*; Magliozzi, S., Ed.; Nova Science Publishers, Inc.: Sfax, Tunisia, 2018; Chapter 3; pp. 101–142.
51. Akbari, M.; Katam, R.; Husain, R.; Farajpour, M.; Mazzuca, S.; Mahna, N. Sodium Chloride Induced Stress Responses of Antioxidative Activities in Leaves and Roots of Pistachio Rootstock. *Biomolecules* **2020**, *10*, 189. [CrossRef] [PubMed]
52. He, L.; Su, R.; Chen, Y.; Zeng, P.; Du, L.; Cai, B.; Zhang, A.; Zhu, H. Integration of manganese accumulation, subcellular distribution, chemical forms, and physiological responses to understand manganese tolerance in Macleaya cordata. *Environ. Sci. Pollut. Res.* **2022**, *29*, 39017–39026. [CrossRef]
53. Ren, S.; Zhou, F.; Xu, C.; Li, B. Simple Method For Visual Detection of Glutathione S-Transferase Activity and Inhibition Using Cysteamine-Capped Gold Nanoparticles as Colorimetric Probes. *Gold Bull.* **2015**, *48*, 147–152. [CrossRef]
54. Erofeeva, E.A. Dependence of Guaiacol Peroxidase Activity and Lipid Peroxidation Rate in Drooping Birch (*Betula pendula* Roth) and Tillet (*Tilia cordata* Mill) Leaf on Motor Traffic Pollution Intensity. *Dose-Response* **2015**, *13*, 1559325815588510. [CrossRef]
55. Varga, B.; Janda, T.; László, E.; Veisz, O. Influence of Abiotic Stresses on the Antioxidant Enzyme Activity of Cereals. *Acta Physiol. Plant.* **2011**, *34*, 849–858. [CrossRef]
56. Jouili, H.; Bouazizi, H.; El Ferjani, E. Plant Peroxidases: Biomarkers of Metallic Stress. *Acta Physiol. Plant.* **2011**, *33*, 2075. [CrossRef]
57. Voet, D.; Voet, J.G. *Biochemistry*, 3rd ed.; John Wiley & Sons: New York, NY, USA, 1990; p. 1223.
58. Busi, M.V.; Gomez-Casati, D.F.; Martín, M.; Barchiesi, J.; Grisolía, M.J.; Hedín, N.; Carrillo, J.B. Starch Metabolism in Green Plants. *Polysaccharides* **2015**, 329–376.
59. Sigfridsson, K.G.; Bernát, G.; Mamedov, F.; Styring, S. Molecular Interference of Cd^{2+} with Photosystem II. *Biochim. Biophys. Acta Bioenerg.* **2004**, *1659*, 19–31. [CrossRef] [PubMed]
60. Amirjani, M. Effects of Cadmium on Wheat Growth and Some Physiological Factors. *Int. J. For. Soil Eros.* **2012**, *2*, 50–58.
61. Ware, A.; Shaikh, F.; Harke, S. Detection and Comparative Analysis of Amylase Activity from Leaves, Seeds and Stem of Purslane (*Portulacaoleracea*). *Int. J. Adv. Res. Sci. Eng.* **2017**, *6*, 793–798.
62. Thalmann, M.; Santelia, D. Starch as a Determinant of Plant Fitness under Abiotic Stress. *New Phytol.* **2017**, *214*, 943–951. [CrossRef]
63. Kusvuran, S.; Kiran, S.; Ellialtioglu, S.S. Antioxidant Enzyme Activities and Abiotic Stress Tolerance Relationship in Vegetable Crops. In *Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives*; Shanker, A.K., Shanker, C., Eds.; IntechOpen: London, UK, 2016; pp. 481–503, ISBN 978-953-51-2250-0.