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Germination and early seedling growth in four *Plantago* species in response to Zn, Cu and Fe

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Abstract

Heavy metal contamination is an increasingly pressing global ecological concern adversely affecting plant growth. Phytoremediation is an eco-friendly and low-cost approach to help solve this problem by using plants to remove metals. This study aimed to evaluate the phytoremediation potential of four *Plantago* species, exposing them to different concentrations (0, 150, 300, 600 and 900 ppm) of zinc, copper, and iron during germination and early seedling growth. These are generally the phases of the plant life cycle most sensitive to stress. The germination percentage (GP), mean germination time (MGT), radicle, hypocotyl and cotyledons length, biomass, water content and tolerance index (TI) were recorded under controlled conditions. The results indicated that metal-induced stress significantly reduced GP, increased MGT, and inhibited seedling growth with increased metal concentration. The relative toxicity of the tested metals could be ranked as Fe > Cu > Zn. Regarding the *Plantago* species, *P. tunetana* and *P. lanceolata* could be considered highly tolerant, *P. albicans* moderately tolerant, and *P. afra* low-tolerant to metal toxicity during germination. Therefore, *P. tunetana* and *P. lanceolata* present an excellent potential for phytoremediation of metal-contaminated zones.

Keywords: heavy metals, phytoremediation, *Plantago*

Introduction

During the past decades, urbanisation and industrialisation have led to environmental pollution and the accumulation of heavy metals in water and soil in many different habitats. Heavy metals are defined as a group of metals with a density greater than 5 g/cm^3 (1). Although some heavy metals, such as zinc (Zn), copper (Cu), and iron (Fe), are essential micronutrients for plants, they have toxic effects on organisms at high concentrations (2-4). Non-degradable metals can impose physiological stress and negatively affect various plants' physiological and biochemical processes, such as nutrient balance, enzyme activities, antioxidant metabolite synthesis, protein mobilisation, and photosynthesis. Metals can cause long-term deleterious effects on ecosystem health and major limitations for crop performance (5, 6). Metals are naturally accumulated in water and soil, and as contaminants due to human activities. Amongst toxic metals, Zn, Cu, and Fe are pollutants present mostly in free ion forms (Zn^{2+}, Cu^{2+} and Fe^{2+}) (7, 8).

Zinc (Zn) is an essential micronutrient for plant growth. It is a crucial constituent of more than 1200 proteins and metalloenzymes, and acts as a cofactor for several enzymes, such as anhydrases, dehydrogenases, oxidases, or peroxidases (9, 10). Zn plays essential roles in regulating nitrogen metabolism, cell multiplication, photosynthesis, protein biosynthesis, and auxin production, amongst other functions (11). Like other trace metals, high Zn concentrations can lead to Zn toxicity that detrimentally affects vital processes at different levels (12). Zn toxicity is usually caused by geochemical and anthropogenic (i.e., industrial activities, mining, smelting, fertilization, and sewage sludge) activities (13). Common symptoms of Zn toxicity in plants include growth inhibition, repression of root

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elongation due to an inhibition of cell proliferation, alteration of water and nutrient uptake, loss of membrane integrity, disruption of redox homeostasis, reduction of chlorophyll content and subsequent photosynthesis, generation of reactive oxygen species and oxidative stress (14, 15).

Copper (Cu) is an essential plant micronutrient with important roles in plant metabolism, enzyme activity and detoxification processes. Cu is released from several industrial activities and by the excess use of fungicides. Copper-containing fungicides and bactericides are used extensively for disease control in many crops (16). Cu is transported through the xylem bound to amino acids (17). The excess accumulation of Cu can cause toxic effects such as chromosome injury and suppression of photosynthesis (18). When present in the soil in high amounts, Cu causes cytotoxic injury to plants, resulting in chlorosis and inhibition of plant growth (19).

Iron (Fe) is an essential micronutrient involved in several vital plant functions, including photosynthesis, respiration, and chlorophyll biosynthesis, by reducing the levels of light-harvesting complexes (LHCs), electron-transport carriers, and carotenoids such as β-carotene and neoxanthin (20-22). Fe toxicity is not often discussed in plant science, even though it causes severe morphological and physiological disorders, including reduced germination, interference with enzymatic activities, nutritional imbalance, membrane damage, and alteration of chloroplast ultrastructure. It also causes severe toxicity to important biomolecules, which leads to ferroptotic cell death and induces structural changes in the photosynthetic apparatus, which results in retardation of carbon metabolism (23).

Metals are toxic pollutants usually accumulated within tailings of abandoned mines (24). Alongside mining sites, the amount of metal pollutants is 100- to 1000-fold higher than the average concentration in soil (25). The metal release is significantly enhanced when tailings are exposed to air, rain and surface water (26, 27). Once these pollutants are released into the surrounding environment, they negatively affect soil, water, natural habitats, and local biodiversity.

Currently, reducing the increasing metal pollution requires applying environmentally friendly technologies such as phytoremediation. This low-cost, sustainable strategy relies on the ability of some plant species to filter or stabilise contaminated soils and waters (28, 29). Screening for plant species with promising properties for phytoremediation is fundamental (30). Some plant species can grow in heavily contaminated environments (31, 32), although the capacity to tolerate and accumulate metals differs in each plant species (33). Moreover, some plant species show contrasted metal tolerance amongst populations (34). In this sense, populations exposed to high metal loads in mining areas may evolve to show higher metal tolerance (35). The first critical step of a phytoremediation programme is germination and seedling establishment in metal-contaminated soils (28). Even though the seed coat can act as a primary barrier limiting the harmful effects of metals, most seeds and seedlings show a decline in germination and vigour when exposed to metal stress.

Seed germination and seedling rooting are essential stages of the whole plant life cycle and are usually the most sensitive to environmental changes (5). Therefore, studying the responses of plants exposed to contaminants at these stages is essential to understanding the toxicity mechanisms of environmental contaminants on plants, and it is an important research area deserving extensive studies.

The *Plantago* genus is a good model for toxicity bioassays as plants of *Plantago* species can accumulate several metals (36-38). *Plantago* species occur in diverse habitats and are resistant to a wide range of stresses (39, 40). Further, their fast growth and relatively high biomass production make these species appropriate objects of study for investigating their sensitivity to the toxic effects of metals as potential species for phytoremediation. Reports on the effects of heavy metals (Zn, Cu, and Fe) on crops are plenty, but very few works have been performed on *Plantago* species, particularly during the germination stage.

The main objective of this study was to investigate for the first time the effects of increasing Zn, Cu, and Fe concentrations on germination and early seedling growth of four *Plantago* species from metal-polluted (mining regions in Kef) and non-polluted areas to find out whether seed germination and early seedling growth were equally affected by exposure to metals. This work aligns with previous studies that describe the metal composition in the soils of the mining regions in Kef, which represent extreme pollution scenarios in the context of our study (41-44). Studying such extremes is crucial for a comprehensive understanding of the potential impacts of heavy metal pollution on germination and early seedling growth, which in turn is essential for identifying the concentrations beyond which plant establishment becomes challenging, selecting suitable plant species, optimising growth conditions, and assessing the overall feasibility of phytoremediation and ecological restoration of heavily polluted sites. *Plantago tunetana* and *P. albicans* seeds were collected from mining metal-polluted areas, whereas *P. lanceolata* and *P. afra* were collected from non-polluted areas. It was expected that the results of this study could provide insights into the plants' responses to Zn, Cu, and Fe toxicity, thus establishing the potential of these *Plantago* taxa for phytoremediation purposes. We hypothesised that *P. tunetana* and *P. albicans* growing in metal-polluted areas would show higher tolerance to metals during germination and early seedling establishment than *P. lanceolata* and *P. afra*.

Materials and Methods

2.1. Description of the studied species and seed collection sites

In this study, four *Plantago* species were collected from different regions of Tunisia. The geographic locations were recorded by a GPS Model Garmin 72. *Plantago tunetana* Murb. is an endemic perennial species with a very restricted distribution area in mining zones in the mountains in northwest Africa. It has ovate or lanceolate-oblong leaves, an obtuse or short blade with seven sinews and a glabrous spike. Seeds of this species were randomly collected from a roadside in Gouraïa, Kef (35°51'30.39" N; 8°41'04.72" E) at 720 m of elevation above sea level (asl) in degraded soil near an iron mining industry (45).

Plantago albicans L. (White Plantain) is a perennial species recognisable by its silky, hairy aspect and lanceolate leaves with wavy margins. Its rhizomatous basis bears suckers and enables active vegetative multiplication. This species grows in wastelands, slopes, and stony rangelands on dry and sun-exposed soils. It colonises open, arid parts of the Mediterranean Basin and runs southward in North Africa in sub-deserted environments. Its seeds were collected from a Zn and Cu mining station in Sers, Kef (36°5'57.07"N; 8°56'18.10"E) near a copper mine at 572 m elevation asl.

Plantago lanceolata L. (Ribwort Plantain) is a cosmopolitan perennial herb. Its leaves are lanceolate, scarcely toothed, and green year-round. The leaf stems are grouped deeply furrowed on a basal rosette; it grows several erect, leafless, silky, and hairy flowering stems, which are taller than the ovoid inflorescence. This species grows in disturbed sites, abandoned crop fields and lawns and is recommended as a bioaccumulation indicator that can accumulate metals in large quantities without visible damage (39, 47, 48). Its seeds were collected from a fig orchard (*Ficus carica* L.) located in Makthar, Siliana (35°49'36.92 "N; 9°21'22.40 "E) at 972 m asl.

Plantago afra L. (sand plantain) is an annual glycophyte species with a wide geographic distribution. It typically reaches 30 to 60 cm in height. Its erect stem bears a cylindrical spike inflorescence containing small, inconspicuous, greenish flowers. The fruits are capsule-like, housing numerous ovate, compressed seeds. *P. afra* is well adapted to semi-arid and arid regions (48). The medicinal properties of *P. afra* have made it an important species in traditional medicine and pharmacology, cultivated in India, Pakistan and Iran. Its seeds were collected from a sand quarry in Bou Argoub, Nabeul (36°28'34.84"N; 10°36'46.35"E) at 152 m of elevation.

2.2. Plant material

Seeds were collected from their natural habitats, as indicated above, in the summer of 2021 from more than 100 randomly selected plants per each studied species. Seeds from different plants were mixed, cleaned, dried, and stored in paper bags at 4 °C in a seed storage room before the germination experiments.

2.3. Germination and seedling growth assays

All experiments were conducted during August and September 2021 in the Higher School of Agriculture of Mograne (36°25'41.32"N–10°5'31.93"E), Zaghouan province, Tunisia. Before being subjected to the germination tests, seeds of the *Plantago* species were surface sterilised in 5% sodium hypochlorite for 5 min to prevent any fungal contamination and then thoroughly washed with sterile distilled water. Afterwards, 25 seeds per species and treatment were placed on 9 Ø cm glass Petri dishes previously autoclaved and containing two layers of autoclaved Whatman No. 1 filter paper moistened initially with 5 ml of the corresponding treatment solution. Petri dishes were sealed with adhesive tape (Parafilm™) to avoid desiccation. Stress treatments were imposed by adding solutions containing 0 (control), 150, 300, 600 and 900 ppm of Zn as $ZnSO_4\,7H_2O$, Cu as $CuSO_4 7H_2O$, and Fe as $FeSO_4 7H_2O$. The metals and these concentrations were chosen based on prior studies that described the metal composition in the soils of the mining regions in Kef, Tunisia, and highlighted the high content of iron, zinc and copper in these regions (41-44). For each species, four replicates of 25 seeds were used. The experiments were conducted in a germination cabinet (Köttermann 2771, Uetze-Hänigsen, Germany) at 25 ± 2 °C in dark conditions. Germinated seeds were counted daily until no further germination was observed for 3 to 4 days (49).

2.4. Studied parameters

Several parameters were determined to assess the effect of Zn, Cu, and Fe stress on seed germination and early seedling growth. Final germination percentage (GP) and mean germination time (MGT) were calculated, the latter using the following formula (50):

$$\text{MGT (days)} = \Sigma\, d.n / \Sigma\, n,$$

Where d is the number of days from the beginning of the germination test and n is the number of seeds newly germinated at day d.

At the end of the experiments, we recorded radicle length (RL), hypocotyl length (HL) and cotyledon length (LA) using an electronic digital calliper and a ruler. Seedlings were weighed in fresh and after being in a forced-air oven at 60 °C for three days. Afterwards, the water content percentage (WC) was calculated as follows:

$$\text{WC (\%)} = [(FW - DW)/FW] \times 100$$

Where FW and DW are the fresh and dry weight of the seedling, respectively.

Finally, the tolerance Index (TI) of the radicle and the cotyledon was calculated using the following formula:

$$\text{TI (\%)} = 100 \times (\text{mean organ length in the treatment / mean organ length in the control})$$

2.5. Statistical analyses

The statistical analyses of the data were performed using SPSS 21.0 (IBM Corporation, Armonk, NY; USA). Throughout the text, all values shown are means of 4 replicas ± standard error (SE). Normality and homogeneity of variance of data series were tested using the Kolmogorov–Smirnov and the Levene tests, respectively. GP, MGT, radicle, hypocotyl, and cotyledon size, fresh and dry weights, and TI (dependent variables) were compared between species, metals, their concentrations, and their interactions (independent variables) using general linear models (GLM). As data did not follow a normal distribution, the results were analysed using generalised linear models (GLZ) with the Wald Chi-square test (χ^2) (51). The effects of each metal on seedling traits of each species were analysed independently using nonparametric Kruskal-Wallis and Mann-Whitney U as *post-hoc* tests.

the indicators of the effect of the heavy metals at concentrations below EC100.

The lower EC50 and EC100 values (higher toxicity) were obtained with iron, followed by copper and zinc. Based on these results, the toxic effect of iron was stronger than the other tested metals, and iron required the lowest concentration to induce the reduction or even absence of the germination of the studied species. Knowledge of the EC50 and EC100 allows us to determine the metal concentration that the species can tolerate.

Table 1. F values (Generalized Linear Models) for the independent factors' Species' (S), 'Treatment' (T) and their interaction 'Species × Treatment' (S × T). Abbr.: GP: germination percentage; MGT: mean germination time; RL: radicle length; HL: hypocotyl length; CL: cotyledon length, DW: dry weight of early seedling; WC: water content of early seedling

	Zn						
	GP	MGT	RL	HL	CL	DW	WC
S	14.48**	13.92**	6.60**	169.84**	7.44**	30.24**	55.61**
T	29.40**	44.56**	716.84**	284.90**	147.19**	36.77**	108.09**
S*T	0.59	3.62**	24.65**	11.11**	36.75**	2.67**	6.79**
	Cu						
	GP	MGT	RL	HL	CL	DW	WC
S	20.38**	14.47**	77.84**	77.75**	135.72**	25.11**	51.39**
T	63.73**	101.38**	566.11**	518.23**	456.68**	87.96**	180.99**
S*T	1.39**	5.20**	22.84**	16.17**	36.32**	5.96**	4.23**
	Fe						
	GP	MGT	RL	HL	CL	DW	WC
S	37.37**	9.56**	162.18**	125.76**	155.63**	39.75**	155.46**
T	78.41**	108.86**	1089.49**	1141.18**	454.17**	61.14**	193.39**
S*T	3.18**	4.09**	42.22**	29.73**	61.18**	8.53**	7.46**

Note: ** $p < 0.01$

Results

Both factors (species and metal treatment) and their interaction showed a significant effect ($p < 0.01$) for all measured parameters (Table 1).

3.1. Metals effects on germination

The effective concentration is constructive in determining the lowest dissolved metal concentration, which reduces seed germination by 50% and 100% (EC50 and EC100).

As shown in Figure 1, there was a significant linear correlation between the reduction of germination percentage of the four studied Plantago species and the tested concentrations of Zn, Cu and Fe.

The EC50 and EC100 (Table 2) were calculated according to the Multiple Linear Regression (MLR) shown in Fig. 1, based on the inhibition of germination. Excessive concentrations of metals in the environment are known to be toxic to plants and can have detrimental effects on seed germination. The dissolved metal concentration measurements were effective as

Regarding species, *P. tunetana* exhibited the highest tolerance of the investigated species, followed by *P. lanceolata* and *P. albicans*, whereas *P. afra* was the most sensitive to the metals. All tested concentrations (up to 900 ppm), whatever the metal, significantly ($p < 0.05$) reduced GP (Fig. 2A) and increased MGT (Fig. 2B). Seeds at control conditions exhibited the maximum GP and the minimum MGT for the four analysed *Plantago* species (Fig. 2A and B). For *P. tunetana*, GP decreased by ca. 16%, 45% and 31% at 900 ppm of Zn, Cu and Fe, respectively, compared to control conditions (Fig. 1A). The corresponding reductions in GP in the presence of 900 ppm Zn, Cu and Fe solutions were 24%, 27% and 49%, respectively, for *P. albicans*; 30%, 34% and 37% for *P. lanceolata*; and 33%, 46% and 84% for *P. afra*. Therefore, in agreement with the EC50 and EC100 data shown in Table 2, *P. tunetana* appeared to be the most tolerant to metals during germination, and *P. afra* the most sensitive species, and Fe was the metal with the most potent inhibitory effect on germination (Fig. 2A).

The progressive reduction of GP with increasing metal

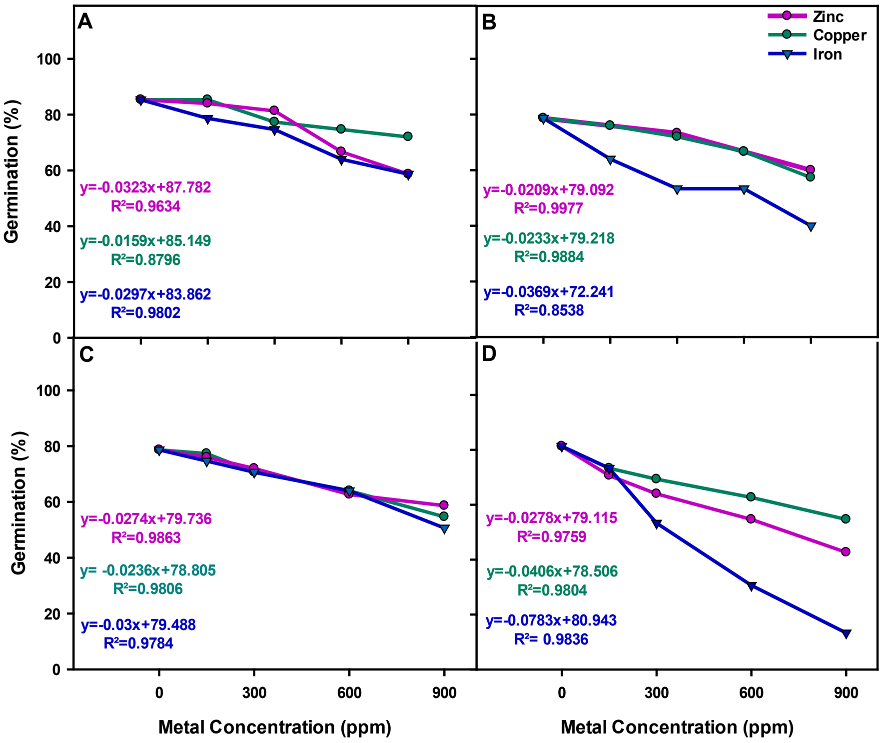

Figure 1. Inhibitory effect of Zn, Cu and Fe on seed germination of *P. tunetana* (**A**), *P. albicans* (**B**), *P. lanceolata* (**C**) and *P. afra* (**D**)

Table 2. The EC50 and EDC100 for zinc, copper and iron, of the germination percentage for each studied species

	Zinc		Copper		Iron	
	EC50 (ppm)	EC100 (ppm)	EC50 (ppm)	EC100 (ppm)	EC50 (ppm)	EC100 (ppm)
P. tunetana	2209.49	5351.47	1168.74	2712.58	1138.15	2824.95
P. albicans	1390.43	3779.42	1252.26	3396.06	603.52	1958.15
P. lanceolata	1083.58	2906.57	1220.76	3335.38	981.60	2648.27
P. afra	1045.32	2843.97	702.70	1931.03	396.14	1034.01

concentrations was accompanied by a parallel increase in MGT. For example, the MGT recorded for *P. tunetana* increased from 5.25 days in control conditions to 6.84, 7.57 and 8.33 days at 900 ppm Zn, Cu and Fe, respectively (Fig. 2B). The corresponding MGT values for *P. afra* were 2.62 days in the control, and 7.33, 8.09 and 8.89 days at 900 ppm of Zn, Cu and Fe, respectively (Fig. 2B).

3.2. Metal effects on early seedling growth

The radicle, hypocotyl, and cotyledon lengths decreased significantly ($p < 0.01$) with increasing concentrations of all tested metals (Fig. 3A, B and C).

For example, germination in the presence of 900 ppm of the tested metals reduced radicle length in all four species between 82% and 96% compared to control conditions (Figure 3A). In *P. tunetana* seedlings, the radicle length decreased by ca. 95%, 94% and 88% upon Zn, Cu, and Fe treatments, respectively. Similar values were recorded for *P. albicans* and somewhat lower, between 82% and 85% reduction, for *P. lanceolata*. *Plantago afra* showed percentages of radicle length reduction of 92% for Zn and about 96% for Cu and Fe (Fig. 3A).
Inhibition of seedling growth by Zn, Cu and Fe was also reflected in a significant, concentration-dependent reduction of hypocotyl length, ranging from 57% for Zn in *P. lanceolata* seedlings and 95% for Cu in *P. afra* seedlings at 900 ppm (Fig. 3B).

Cotyledon length was also significantly reduced by metal treatments, although to a lesser extent than the radicle or the hypocotyl, except for Zn in *P. lanceolata* seedlings. Again, the strongest reductions compared to the control were observed in *P. afra*, amounting to 67% for Zn, 83% for Cu, and 85% at 900 ppm Fe. Given these results, *P. afra* appeared to be the most

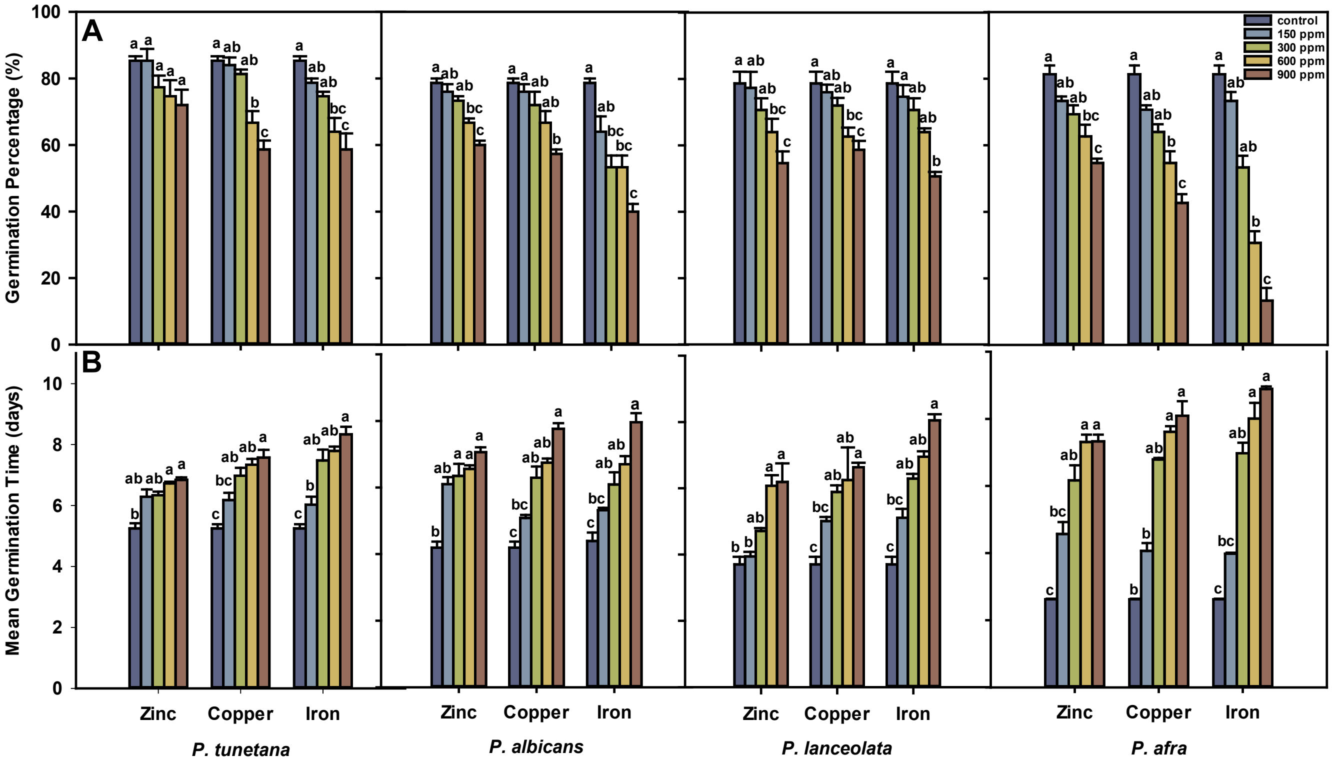

Figure 2. Germination percentage (A) and Mean Germination Time (MGT) (B) for *Plantago tunetana*, *P. albicans*, *P. lanceolata* and *P. afra* seeds treated with different concentrations of zinc (Zn), copper (Cu) and iron (Fe). Data shown are means ± SE (n = 4). Different letters indicate significant differences between concentrations for each metal and species (Mann-Whitney U test, $p < 0.05$).

sensitive species to metals, and Fe was the most toxic element (Fig. 3C), as determined considering germination percentages and mean germination times (Fig. 2).

Early seedling growth also showed a significant reduction ($p < 0.01$) in biomass (dry weight) with increasing in metal concentrations, although with quantitative differences between species and metals (Fig. 4). For example, *P. tunetana* seedlings treated with 900 ppm Zn, Cu or Fe showed similar biomass reductions, 40-44% in relation to control conditions, whereas the same metal concentrations in *P. albicans* caused a decrease of biomass of 44% for Zn and 67% for Cu and Fe. The strongest biomass reduction (84%) was observed for *P. afra* seedlings treated with 900 ppm Fe (Fig. 4A).

Metal treatments also led to the dehydration of the seedlings of all analysed species. Their WC decreased from values above 80% in control conditions to 40% or less in the presence of 900 ppm Zn, Cu or Fe (Fig. 4B).

The radicle and cotyledons tolerance index of the four *Plantago* species treated with different concentrations of Zn, Cu and Fe are presented in Fig. 5. The results show variable changes depending on the species and the metal but maintain a general decreasing trend with increasing metal concentrations. The root tolerance index appeared more sensitive to metal toxicity than the cotyledon tolerance index. Tolerance indices were generally higher for seedlings treated with Zn, followed by Cu and Fe. *Plantago lanceolata* showed the highest root tolerance index in seedlings treated with 900 ppm of Zn, Cu and Fe compared to the other studied species. *Plantago tunetana* and *P. albicans* did not show substantial differences in their root tolerance indices, but *P. tunetana* showed a higher cotyledon tolerance index than *P. albicans*. *Plantago afra* was the most sensitive species, showing the lowest root and cotyledon tolerance indices (Fig. 5).

Discussion

The results of the present study revealed that Zn, Cu, and Fe limited germination for the four tested *Plantago* species, as shown by a gradual and significant decrease in GP and an increase in MGT. In all cases, the observed effects of metals on germination were concentration-dependent, although Zn, Cu and Fe showed different degrees of toxicity. In plant ecophysiology, the establishment of the toxicity ranking order of metals is complex and remarkable differences can be noticed depending on *i*) the metal (chemical characteristics and applied dose), *ii*) the studied plant (species, development stage) (52), and *iii*) the experimental approach (duration and conditions of the treatments, plant substrate) (53). Comparing the inhibitory effect of the three tested metals on germination and early seedling growth, their relative toxicity could be ranked as Fe > Cu > Zn, which is more or less in line with previous research.

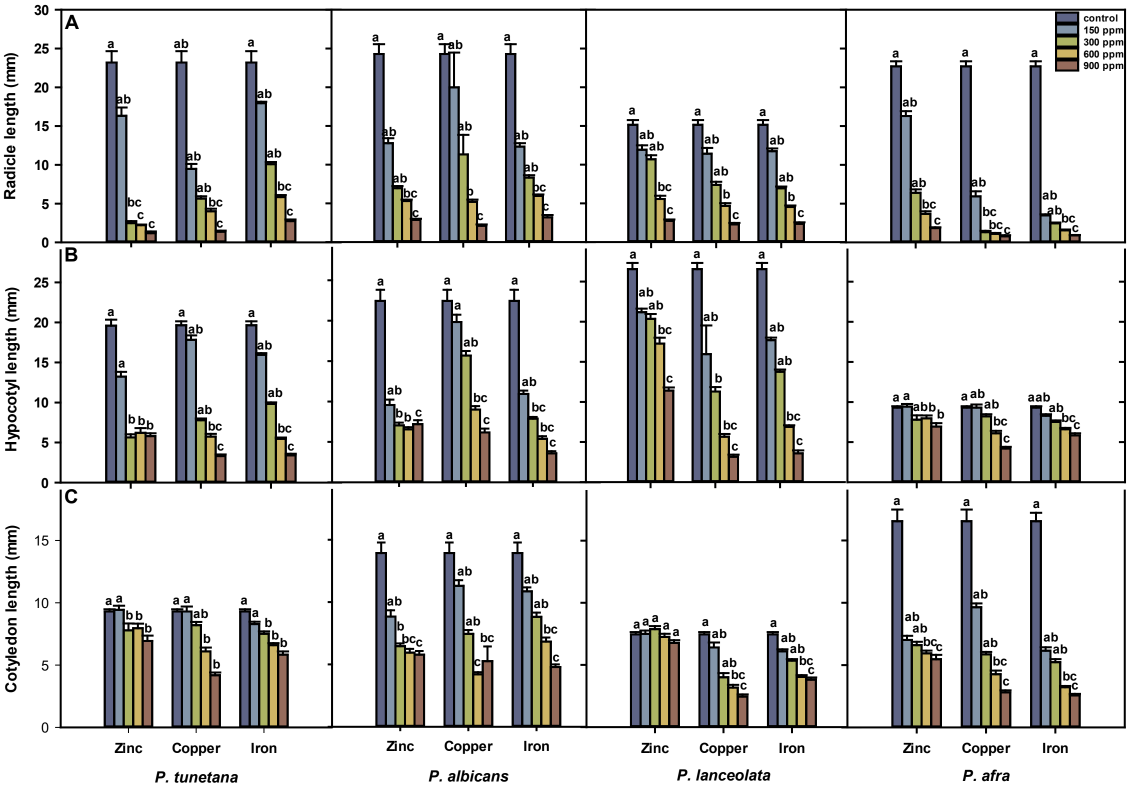

Figure 3. Size (mm) of radicle, hypocotyl, and cotyledons for *Plantago tunetana*, *P. albicans*, *P. lanceolata* and *P. afra* seeds treated with different concentrations of zinc (Zn), copper (Cu) and iron (Fe). Values are mean ± SE (n = 4). Different letters indicate significant differences between treatments for a given organ and species (Mann-Whitney U test, $p < 0.05$).

For example, the relative inhibitory effect of several metals on *Medicago sativa* L. germination has been reported as Cd > Cu > Ni > Pb > Zn (54). In *Medicago arborea* L., the relative toxicity of selected heavy metals on seed germination was ranked as Cu > Pb > Zn (53).

A similar inhibitory effect of germination by metals as the one recorded in our study has been reported previously for several species belonging to different plant families, for example, *Cicer arietinum* L. (55) or *Medicago sativa* (56). The four tested *Plantago* species could germinate at metal concentrations as high as 900 ppm, whereas germination of *Arabidopsis thaliana* (L.) Heynh. was completely inhibited by 200 mM Cu^{2+} (57). Many studies have demonstrated that Zn does not affect seed germination (58). However, the increase of Cu and Fe supplies exerts a significant inhibitory effect on seed germination (59, 60). Our results support the idea that tissues covering the embryo play a key role in the selective penetration of different metals into seeds, representing a highly protected part of a plant's life cycle, and that the seed coat is an important defence against abiotic stress (61). This was first suggested by the fact that seeds still germinated in the presence of high concentrations (900 ppm) of Zn^{2+}, Cu^{2+}, and Fe^{2+}, but the subsequent seedling growth was severely inhibited at lower concentrations of these metals (300 and 600 ppm) (62). As in other studies, the seedling growth phase was more sensitive to metal exposure than seed germination (63, 64), which was reflected in drastic decreases in biomass and the length of radicle, hypocotyl and cotyledons with increasing metal concentrations for all tested species. Metal effects caused structural and morphological changes in roots and the inhibition of root hair growth of seedlings before shoot growth (65). An excess of Zn inhibited cell division in root tips, causing a reduction in growth (66). It also caused chlorosis in young leaves, inhibited photosynthesis (67) and affected the uptake of other nutrients (68). In *Beta vulgaris* L. plants, a high excess of Zn induced a decrease in the steps of aerobic respiration, a reduction in root growth and the display of a brown colour with short lateral roots (69). Some studies revealed that prolonged exposure to Cu-stress of *Allium* species, barley and *Raphanus sativus* inhibited root elongation and caused shoot necrosis (70-72). Regarding Fe, a significant decrease in wheat root and shoot length was reported at 1000 ppm of iron when compared with the control (73). In general, the radicles of the studied *Plantago* species were more affected by an increase in Zn, Cu, and Fe doses than the cotyledons. Iron toxicity can result in the presence of leaf chlorosis, darkening of roots (74), and reduction of the total water content (75), leading to reduced plant growth (76). The results regarding radicle and cotyledon tolerance indices indicated that the lowest concentrations of these metals induced an increase in tolerance, whereas high

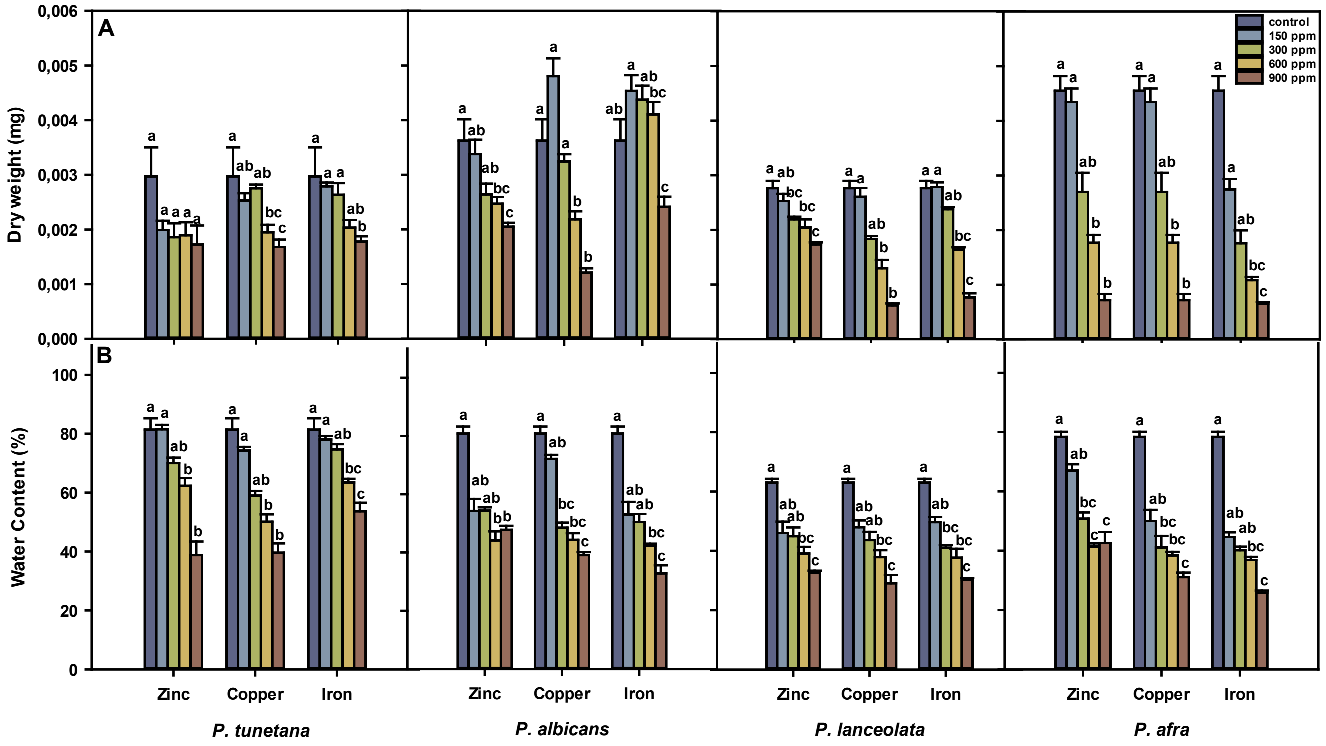

Figure 4. Seedling dry weight and water content of *Plantago tunetana*, *P. albicans*, *P. lanceolata* and *P. afra* seeds treated with different concentrations of zinc (Zn), copper (Cu) and iron (Fe). Values are mean ± SE (n = 4). Different letters indicate significant differences between treatments for a given parameter (Mann-Whitney U test, $p < 0.05$).

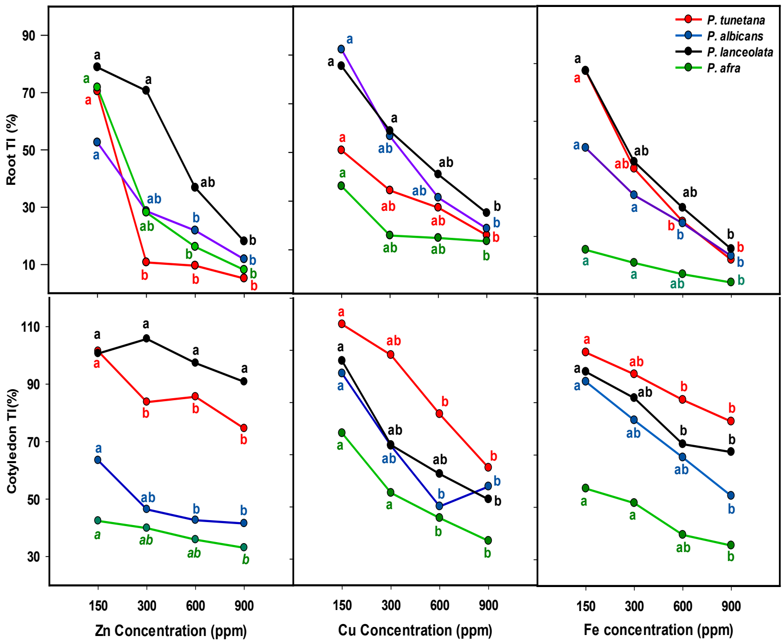

Figure 5. Radicle and cotyledon tolerance indices (TI) for four *Plantago* species treated with different concentrations of zinc (Zn), copper (Cu) and iron (Fe). Values are mean ± SE (n = 4). Different letters indicate significant differences between treatments for each metal and species (Mann-Whitney U test, $p < 0.05$).

concentrations decreased this parameter. These findings were in concordance with previously published results (77, 78).

As we hypothesised, the endemic species *P. tunetana*, coming from a mining metal-polluted site, was more tolerant to metals than the other tested species. The relatively high metal tolerance of the metallophyte *P. lanceolata* and the endemic *P. tunetana* seedlings in comparison to the glycophyte *P. afra* may be explained by their different habitats since *P. tunetana* is a coloniser of areas of iron mining, where metal pollution is higher than at areas colonised by *P. afra*. *Plantago tunetana* would have had more time than the other *Plantago* species to adapt to the polluted environment of north-western mined areas in Tunisia. In view of our results, *P. tunetana* and *P. albicans* and *P. lanceolata* could be suitable species for phytoremediation since they can germinate and establish in highly polluted conditions.

Conclusions

Drainage from abandoned mines is a significant environmental concern and usually contains appreciable concentrations of heavy metals. The knowledge of metal toxicity levels for seed germination is essential for the proper functioning of the treatment system for mine drainage. Considering the overall responses of germination and early seedling growth of the selected *Plantago* species to metals, their relative toxicity can be classified as Fe > Cu > Zn. Regarding the relative tolerance of these species, *P. tunetana* and *P. lanceolata* were highly tolerant to all tested metals, followed by the moderately tolerant *P. albicans*, whereas *P. afra* was a sensitive species. It is noteworthy that the *Plantago* seeds could germinate even at 900 ppm Zn, Cu or Fe, which is a concentration much higher than the critical limits for agricultural soils and irrigation water. This suggests that *P. tunetana*, *P. lanceolata,* and, to a lesser extent, *P. albicans* are species with great potential for phytoremediation of metals.

Author Contributions

Conceptualization, H.B.L, A.S and S.R.; methodology, H.B.L.; A.S. and S.R. software, H.B.L., A.S. and J.M.C.; validation, J.M.C., S.R. and O.V; formal analysis, investigation, resources, data curation, H.B.L. and A.S.; writing—original draft preparation, H.B.L. and A.S. writing—review and editing, J.M.C, S.R. and O.V.; visualization, J.M.C. and S.R.; supervision, S.R. and O.V.; funding acquisition, S.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data are contained within the article.

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Conflicts of Interest

The authors declare no conflict of interest.

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