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Greenhouse investigation on the phytoremediation potential of pioneer tree Pinus halepensis Mill. in abandoned mine site

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Ex-situ Investigation on Phytoremediation Capability of *Pinus halepensis* Mill. a Pioneer Tree in Abandoned Mine Tailing

Abstract

Tailings and mine dumps are often pollutant sources that pose serious environmental threats to surrounding areas. The use of pioneer vascular plants to extract or stabilize metals is considered among the more effective mine tailing reclamation techniques. The study aimed at evaluating the phytoremediation potential of *Pinus halepensis* in abandoned mine-tailing (SW-Sardinia, Italy). Plant ability to tolerate high Zn, Pb, and Cd concentration and their accumulation in roots and aerial parts were assessed at greenhouse conditions. Experiments were performed on 45 seedlings planted in different substrates (mine-tailings, mine-tailings compost-amended, and reference) and on 15 seedlings grown spontaneously in the contaminated mine site investigated with their own substrates. The phytostabilization potential of plant was evaluated through biological accumulation and translocation indexes together with plant survival and biometric parameters. The outcomes showed the adaptability of *P. halepensis* to grow and survive in contaminated substrates. Compost addition did not improve plant survival and growth, however, it enhanced total carbon and nitrogen contents of soil, restricted metal bioavailability, and accumulation in plant aerial parts. These findings highlight that *P. halepensis* may be considered for phytostabilization given the great potential to limit Zn, Pb, and Cd toxicity in plant tissues by applying compost amend ment in metal contaminated mine sites.

Keywords: metal tolerance; phytoremediation; phytostabilization; *Pinaceae*; translocation indexes; biometric parameters

Novelty Statement

The novelty of this study is the selection of *Pinus halepensis* Mill. as a proper tree species for long term phytoremediation of multi-heavy metal mine tailing sites. This plant species not only had adaptation to the Mediterranean climate and could tolerate high temperatures and high metal concentrations, but also showed high survival and growth percentage of its roots and epigean organs in highly contaminated mine tailing. *Pinus halepensis* could limit metal accumulation and toxicity in the aerial part of the plant with the addition of compost amendment. This study demonstrates that compost can enhance soil properties and modify metal bioavailability. The outcomes can be beneficial for the phytostabilization project and restoration of similar Mediterranean mine sites.

Introduction

Tailings are the most important sources of toxic heavy metals in mine sites. The exposure of fine-grained minerals to atmospheric agents makes them subjected to oxidative dissolution processes (Mendez and Maier 2008). Metals can be mobilized through aeolian dispersion and surface runoff and mostly spread a high range of contaminants in soils and ground waters of surrounding areas (Concas et al. 2006; Cao et al. 2008; Barbafieri et al. 2011; Bacchetta et al. 2015). For this reason, mine areas are often affected by low vegetation survival and growth, which may result in the loss of biodiversity due to the high content of heavy-metals and the low nutrients and organic carbon in soil (Baker et al. 2010; Ahirwal and Pandey 2021). These unfavorable conditions are common environmental challenges in most of heavily-contaminated mine areas and require effective sustainable remediation.

The pioneer plant species growing spontaneously in these highly-polluted environments are sufficiently resilient, well-adjusted to the local climate and can tolerate high concentrations of metals (Cao et al. 2009; Concas et al. 2015; Lai et al. 2015; Bacchetta et al. 2018). Some of these autochthonous plant species can be effective and beneficial for *in-situ* reclamation of mine tailing sites utilizing phytostabilization processes (Mendez and Maier 2008; Lai et al. 2015; De Giudici et al. 2015; Medas et al. 2017; Conesa and Párraga-Aguado 2019). In general, phytostabilization is considered an appropriate technique because through its bio-mineralization processes, it helps the low accumulation of metals in the aerial parts of plants and the long-term establishment of plant canopy in mine wastes (Conesa and Párraga-Aguado 2019; Monaci et al. 2020). This can be improved by appropriate soil-amendments capable of contributing to the recovery of organic matter and essential nutrient elements (N, P, K, Cr, Cu and Zn) in soil, enhancing water retention, improving root penetration and plant growth (Barbafieri et al. 2011). Among the investigated soil-amendments, compost seems to play an important role in optimizing the phytoremediation process by lowering the metal bioavailability and its accumulation in plant tissues (Fagnano et al. 2011; Bacchetta et al. 2015; Conesa and Párraga-Aguado 2019).

Southwest Sardinia (Italy) has a rich history of mine activities dated back to the pre-Roman time. The mine activities together with the related inadequate actions performed to alleviate metal impacts after mine closure have left a high content of metal pollutants in mine wastes estimated to be at about 70 million m^3 (Jiménez et al. 2014; Bacchetta et al. 2018). Several pilot project studies have been carried out in Sardinian abandoned mining areas availing of its rich biodiversity in order to identify the appropriate autochthonous plant species performing the best phytoremediation capabilities (e.g., Concas et al. 2006; Cao et al. 2008; Barbafieri et al. 2011; Bacchetta et al. 2012). The metal tolerance adaptability has been investigated as well as the survival capability of several Sardinian grasses, half-shrubs and shrubs growing spontaneously on the highly-contaminated mine waste, such as *Pistacia lentiscus* L., *Scrophularia canina* L. subsp. *bicolor* (Sibth. et Sm.) Greuter, *Dittrichia viscosa* L. Greuter, *Cistus salviifolius* L., *Euphorbia cupanii* Bertol. ex Moris, *Helichrysum microphyllum* Cambess. subsp. *tyrrhenicum* Bacch., Brullo & Giusso, *Phragmites australis* (Cav.) Trin. ex Steud, *Euphorbia cupanii* Bertol. ex Moris and *Juncus acutus* L. (e.g., Jiménez et al. 2011, 2021; Bacchetta et al. 2012, 2015, 2018; De Giudici et al. 2015; Medas et al. 2015, 2017; Lai et al. 2015; Boi et al. 2019, 2020, 2021). However, few available findings investigated the use of woody tree species despite their potential for long-term stabilization of mining sites (Disante et al. 2010; Conesa and Pàrraga-Aguado 2019).

Pinus halepensis (Aleppo pine), is a circum-Mediterranean tree species (Tutin et al. 1993; Fady et al. 2003; Farjon 2017; Pesaresi et al. 2017; Pignatti 2017–2019) that can grow spontaneously in different substrates often in marls, limestone (Barbéro et al. 1998; Mauri et al. 2016; Farjon 2017) and low fertile soil at the altitudinal range from sea level up to 1700 m a.s.l. in Morocco

(Farjon 2017). It is a thermophilous and xerophilous plant that grows in areas with annual average rainfall between 350 and 700mm (Barbéro et al. 1998; Mauri et al. 2016) and tolerates temperatures below 10°C (Querejeta et al. 2008). In Mediterranean Basin, the forests dominated by this species cover more than 3.5million ha but despite the species name coming from Aleppo town in Syria mainly in the western part, they are more scattered in the eastern side (Pesaresi et al. 2017). In Italy, *P. halepensis* is considered a native species in many regions, as well as the islands of Sicily and Sardinia (Bartolucci et al. 2018 and 2020). The usefulness of *P. halepensis* in the restoration of degraded arid and semi-arid soils is documented in the literature, because of its metal tolerance and eligibility to survive in low-nutrient conditions (Querejeta et al. 2008; Pàrraga-Aguado et al. 2013). These findings make this plant a suitable candidate for performing phytoremediation experiments. In this study, in order to better assess the phytoremediation of *P. halepensis*, one of the widespread evergreen tree species growing on the Mediterranean mine sites, the experiment was carried out under the greenhouse-controlled condition.

The aims of this study were to assess the capability of *P. halepensis* to tolerate high concentrations of Zn, Pb and Cd, in terms of: (**i**) plant survival and growth (elongation and biomass (dry weight) production of roots and epigean organs); (**ii**) metals bioavailability and its accumulation in roots and aerial parts of the plant through the evaluation of biological parameters and (**iii**) mitigation of heavy metals stress in plant by assessing the effectiveness of compost-organic amendment.

Materials and Methods

Study area

The study area is Campo Pisano mine tailing (CP) located in the Iglesiente biogeographic subsector (Fenu et al. 2014), South-Western Sardinia, Italy (Figure 1). The area is characterized by a Mediterranean pluviseasonal bio-climate ranging between the upper thermo- and the lower meso-Mediterranean (Bacchetta et al. 2009), within 600 mm annual mean precipitation, 17°C temperature, around 57% evapotranspiration and 24% runoff (Cidu et al. 2001; Lai et al. 2015). The surface layer of CP mine waste is generally classified as neutral or slightly alkaline and sandy-loam based on the USDA classification (USDA 1998), while the deep layers are characterized differently and are more acidic due to the metal-sulfides and pyritic-rich materials (Aversa et al. 2002; Kharazian et al. 2022b). The different mine extraction methods during the exploitation activities caused the intensive heterogeneity of metal concentrations and poor nutrient contents in CP mine wastes (Boni et al. 1999). In this study, the mine waste samples were collected in the CP mine site (Figure 1) at a maximum depth of 30cm, transported to the laboratory, mixed and homogenized several times with a shovel.

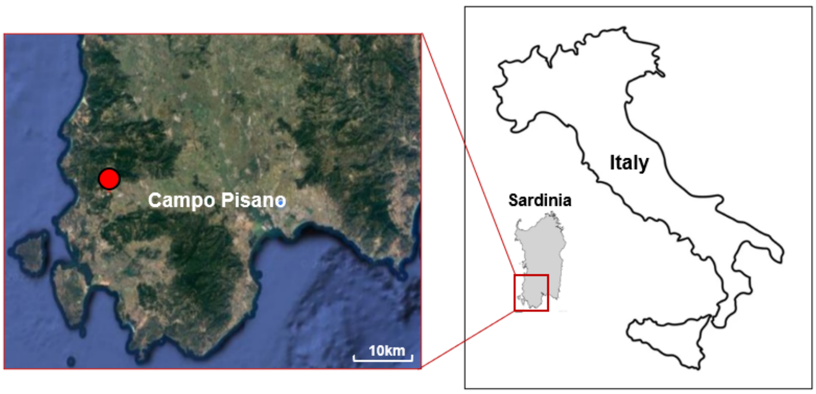

Figure 1. Location maps of the study area in south-west of Sardinia, Italy; the red sign indicates the sampling site of Campo Pisano mine tailing dumps

Experimental Design

The selection of *P. halepensis* was based on the preliminary study of plant made by Kharazian et al. (2022a and 2022b), its adaptability to the local geochemical and mineralogical characteristics, climate and its option to grow spontaneously in this highly polluted environment.

Forty-five healthy seedlings of *P. halepensis* (roughly 4–6 months old) provided by the Bagantinus plant nursery (PN) of the "Forestas Agency", "Regione Autonoma della Sardegna" (Decimomannu, South Sardinia), have been selected (out of approximately 100 similar specimens) and placed for some days into the greenhouse to be adapted to the new environment.

Thereafter, these seedlings were transplanted randomly and individually in three distinct sets of 15 pots (1.7L-polyethylene pots; 16×13×10cm). The pots of each set were filled with 1.5L of specific homogenized substrates: (**i**) not-contaminated reference treatment (NOC) consisting of 45% peat, 45% sand and 10% commercial soil; (**ii**) Campo Pisano contaminated mine waste (CP), and (**iii**) Campo Pisano mine waste amended with 30% w/w of compost from Municipal Solid Waste (MSW) organic fraction (CPC) (Figure 2), provided by "Tecnocasic S.p.A." (Cagliari, Italy). Moreover, fifteen *P. halepensis* seedlings (about 3–6 months-old) spontaneously grown in Campo Pisano contaminated site (PS), were also collected randomly (out of approximately 50 similar specimens), transferred into similar pots and filled with about 1.5L of their own CP-contaminated substrate (**S**).

All pots were placed into the greenhouse laboratory of Sardinian Germplasm Bank (BG-SAR, Cagliari) (Porceddu et al. 2017) for being investigated under controlled conditions (constant temperature at 20°C, humidity at 60–65%, and 12hours-light/ 12hours-darkness as photoperiods) for six months (Figure 2). Plants were watered twice a week with 30mL of distilled water for the first two months and continued 3times a week with 50mL until the end of experiment. The amount of water was based on the plant needs and according to the findings of previous phytoremediation lab-scale experiments carried out on the Mediterranean plant species (Bacchetta et al. 2015; Boi et al. 2021). Before greenhouse trials began (time T0), homogenized substrates and plants were characterized. The total Zn, Pb and Cd concentration and their bioavailable fraction were assessed in the four selected substrates (NOC, CP, CPC, S). As for plant specimens, five seedlings of pines (out of 100 similar specimens) from the nursery (PN) and five seedlings of pine (out of 50 similar specimens) grown naturally in Campo Pisano site (PS) were randomly selected at the same time of selecting plants for planting in ports, washed with distilled water and dried at 45°C. Each specimen was split into roots and epigean organs and characterized in terms of length and biomass dry weight (Figure 2). The accumulation of Zn, Pb and Cd was measured in a single composite sample (out of five samples) for root and epigean organs separately. All these parameters were evaluated again at the end of six months experiment (T6), by removing plants from the pots and washing them before analyses.

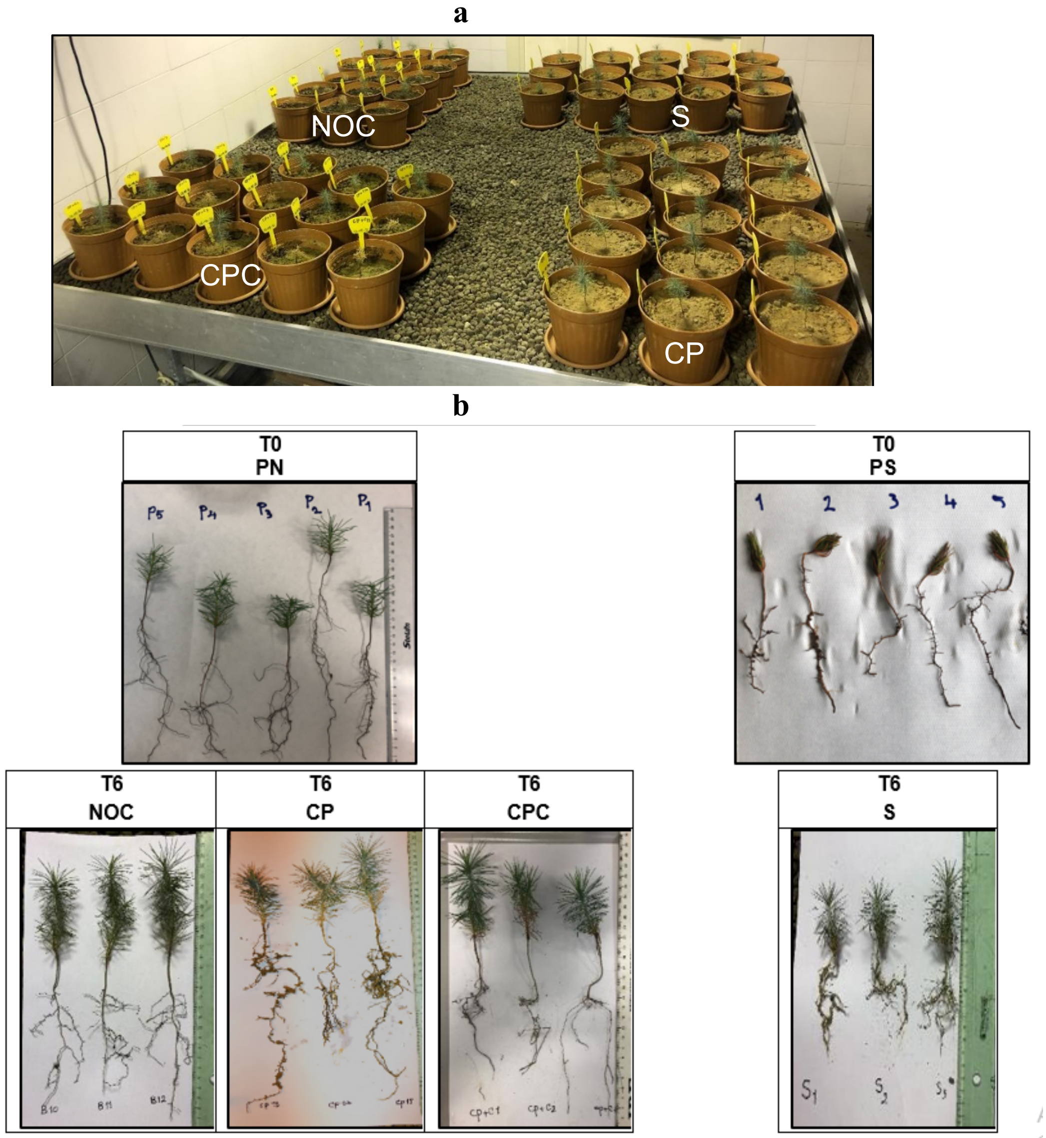

Figure 2. The lab scale experiments on *Pinus halepensis* samples in different substrates. (**a**) Pots of the considered treatments: seedlings from nursery planted in not-contaminated reference (NOC); seedlings from nursery planted in Campo Pisano substrate (CP); seedlings from nursery planted in CP substrate amended with compost (CPC); contaminated substrates with its plant samples collected from the CP mine site (S). (**b**) Seedlings from the nursery (PN); seedlings from the contaminated site (PS), before planting (T0) and after six months (T6) are shown as representative of collected plant samples.

Substrate Analysis

Physico-Chemical Characteristics of Substrates

The substrate of each treatment (15pots) was mixed and homogenized in a single composite sample, sieved (<2mm), ground in fine powder in an agate mortar and oven-dried at 45°C (Binder GmbH, Tuttlingen, Germany). The chemical characteristics of substrates were analyzed following the Italian society protocol of the soil science D.M.13.09.1999 (Barbafieri et al. 1996; Lindsay and Norvell 1978). Soil pH was measured in distilled water (1:2.5) using a pH-meter (ORION model 230A). The Electrical Conductivity (EC) of substrates was calculated in 50mL distilled water (1:1) utilizing a conductivity-meter (HANNA-DiST4). Total carbon and total nitrogen as well as organic carbon (OC) content of the substrates were determined through Micro-analyzer (CHNS/O-2400-SERIE-II PERKIN-ELMER) calibrated with the reference material (ore-minerals). Organic carbon (OC) was analyzed after soil samples were treated and acid-washed (HCl 1mol L^{-1}) to remove carbonate content, according to Ussiri and Lal (2008).

Total Metal Content and Bioavailable Concentration of Metals in Substrates

The total metal concentrations in substrates were assessed using the Environmental Protection Agency (EPA) method 3052 (see details at Bacchetta et al. 2012). It was performed using microwave-assisted acid digestion (Start D, Milestone, Sorisole, Italy) following the procedure according to Bacchetta et al. (2018). Samples were processed with certified reference materials, GBW-07403-GSS-3 for substrates and the blank solution (the EnviroMAT-pure water high reference solution, EP-H-1150) and then both were matrix-matched with reagents and samples. The bioavailable content of Zn, Pb and Cd was evaluated for all substrates through a single extraction method using DTPA solution (diethylenetriaminepentacetic acid), calcium chloride ($CaCl_2$) and triethanolammine (TEA) adjusted to pH~7.3 following the procedure made by Lindsay and Norvell. (1978), Barbafieri et al. (1996) and Feng et al. (2005). This extraction procedure is recognized to be an efficient method to prevent the dissolution of carbonate as well as the release of bounded metals (Feng et al. 2005). The bioavailable fractions of metals (bf) were measured (in percent) considering the mean values of bioavailable fractions of metal (M_{bf}) and the total metal content (M_{total}) in the substrates. $bf(\%)=(M_{bf})/(M_{total})\times 100$ (1)

The total metals content and the bioavailable fractions of the substrate were both determined by Inductively Coupled Plasma Emission Spectrometry (ICP-OES, Perkin Elmer Optima, Waltham, DV7000) after cooling, filtering and diluting the prepared solutions using distilled water (Barbafieri et al. 1996; Guan et al. 2011).

Plant Analysis

Metal Accumulation in Plant Tissues

Before metal analyses, the roots and the epigean organ samples were separated and ground into powder using an electric grinder (Ultra Centrifugal Mill ZM200, Retsch GmbH). Metal accumulation in plant tissues was measured following the same procedure described in Kharazian et al. (2022a). Data quality was also assessed by processing certified reference materials, GBW-07603-GSV-2 (bush-twigs and leaves) and the concentration of metals was analyzed by ICP-OES using the same wavelengths performed for the substrates. Due to the low biomass production of plants at the end of six months (T6), for each treatment, a single composite root sample was produced (out of 15 specimens) as well as three composite samples of aerial parts (out of each 5 samples).

Assessment of the Biometric Parameters: Plant Growth and Phytoremediation Potential

The variation of biometric parameters (BP) was calculated in each treatment at the end of experiment (T6) in comparison with control sample (T0).

$$\text{BP Variation } (\%) = (BP_{T6} - BP_{T0})/BP_{T0}\times 100 \quad (2)$$

The phytoremediation potential of plant to extract metals from the substrate and translocate them to the epigean organs was assessed using the biological indexes, namely Biological Accumulation Coefficient (BAC), Translocation (TF) and Biological Concentration Factors

(BCF). The BAC was measured to estimate metal transferring from substrate (M_S) to epigean organs (M_E) (Marchiol et al. 2013) as $BAC=(M_E)/(M_S)$ (3)

The metal translocation from roots (M_R) into epigean organs (M_E) (Brunetti et al. 2009) was measured as $TF=(M_E)/(M_R)$ (4)

The BCF reported the ratio between metal content in roots (M_R) and substrate (M_S) according to Fellet et al. (2007): $BCF=(M_R)/(M_S)$ (5)

Data Analysis

One-way ANOVA was used to evaluate statistical differences between the plant growth in different substrates and to assess variations of the growth rate from T0 to T6 in terms of elongation and biomass production of root and the epigean organ of *P. halepensis*. Moreover, the significant differences highlighted by ANOVA were afterward analyzed by the post-hoc pair-wised comparisons T-test (Tukey-test). The result values are expressed as the mean±standard deviation and the significant level of statistical analysis in all cases considered $p<0.05$, using Sigmaplot 11.0 software. The matrix of Principal Component Analysis (PCA) was performed to examine the correlations between total metal contents in soil and metal accumulation in plants with the biological parameters and DTPA-extracted metals in contaminated and not-contaminated substrates using the statistical software CANOCO5 proposed by ter Braak and Smilauer (2002).

Results

Substrate's Analysis

Table 1 shows the main physico-chemical properties of soil samples as well as total Zn, Pb and Cd concentration and bioavailable fractions of metals in all substrates. The data shows that the substrates of CP and S were slightly alkaline (pH 7.2–7.8) and presented low EC (3.4–6.8 dSm^{-1}), whereas the compost-amended substrate (CPC) showed acidic (pH 6.1–6.7) and high EC (20.2–21.2 dSm^{-1}). Both CP and S substrates presented low content of organic carbon (upto

0.7%) and nitrogen contents (upto 0.1%). The highest total carbon (upto 13.5%) and organic carbon (upto 2.1%) were reported for CPC substrate.

Table 1. Total metal content (Zn, Pb and Cd) and physico-chemical characteristics of substrates (means ± SD, n=2); NOC: not-contaminated substrate; CP: Campo Pisano substrate with plants from nursery; CPC: Campo Pisano substrate with compost; S: Campo Pisano substrate with plant grows naturally in contaminated site; bf: bioavailable fraction; T0: before planting; T6: after six-months study

	Parameters		NOC	CP	CPC	S
Zn	Zn-total (mg kg^{-1})	T0	59.2±0.9	28041.7±3153.5	20193.9±769	34842.3±2899.3
		T6	50.9±4.6	30038.6±2367.03	21334.9±1257.5	30444.08±4525.3
	Zn-bf (%)	T0	17.4	8.8	0.9	7.3
		T6	16.5	7.9	0.2	8.0
Pb	Pb-total (mg kg^{-1})	T0	18.9±2.9	17270.5±2404.4	13324.5±48.6	24338.01±2242.6
		T6	18.3±2.3	15258.5±2708.5	14859.04±42.5	19644.3±4420.9
	Pb-bf (%)	T0	31.8	6.5	0.2	3.6
		T6	27.7	8.2	0.4	6.3
Cd	Cd-total (mg kg^{-1})	T0	0.92±0.5	121.9±11.6	108.4±0.5	141.4±6.4
		T6	0.37+0.06	106.1±5.1	113.8±0.05	124.9±7.4
	Cd-bf (%)	T0	14.8	10.6	2.1	11.6
		T6	29.4	13.05	1.1	11.5
pH		T0	7.8	7.2	6.1	7.5
		T6	7.8	7.3	6.7	7.8
EC (dSm^{-1})		T0	4.2	4.7	21.2	3.4
		T6	3.1	6.8	20.2	3.5
Total C (%)		T0	4.5±0.4	7.8±0.06	13.5±0.2	6.6±0.2
		T6	5.1±0.9	7.6±0.1	13.2+0.2	6.3±0.1
Total N (%)		T0	0.4±0.05	0.1±0.01	0.3±0.03	0.1±0.02
		T6	0.4±0.06	0.1±0.01	0.3±0.02	0.1±0.01
Organic C (%)		T0	3.6±0.2	0.3±0.02	2.1±0.5	0.7±0.06
		T6	3±0.2	0.3±0.01	1.8±0.2	0.5±0.01

The most abundant metal in all substrates was Zn, followed by Pb and Cd in each treatment. Indeed, the total metal content was high in S and CP substrates with the highest values reported for Zn (upto 34842.3mg kg^{-1}), Pb (upto 24338.01mg kg^{-1}) and Cd (upto 141.4mg kg^{-1}) in S substrate. All metals concentration remained almost constant in each substrate at T6. The total metal content (mainly Zn and Pb) measured in CPC was lower than in CP and S, indicating that compost addition has a diluting effect on mine waste (Table 1).

The bioavailability of all investigated metals in not-contaminated (NOC) substrate was higher than CP, S and CPC substrates (Table 1). Cadmium was the most bioavailable metal in all substrates (T6), except CPC. The lowest bioavailable fraction for Cd was reported in CPC upto 2.1% in T0 which decreased to 1.1% in T6. Zinc (0.9%) and lead (0.4%) were also the lowest bioavailable metals in the same substrate. The bioavailable values for Cd were reported 13.05% in CP and 11.6% in S substrates.

Plant Analysis

The preliminary metal analysis assessed in plant tissues (T0) showed that root and epigean organs accumulated Zn>Pb>Cd which is followed also through the phytoremediation test (T6) in each treatment (Table 2). Metal concentrations measured at T6 were generally higher in roots than epigean parts in all substrates. The highest accumulation of Zn, Pb and Cd in plant tissues was detected in S followed by CP and CPC. In compost-amended substrate (CPC), Zn and Pb were accumulated mainly in root samples (534.7 Zn and 707.3mg kg^{-1} Pb) and their accumulation in epigean organs (24.2 Zn and 21.9mg kg^{-1} Pb) was lower than contaminated substrates (upto 505.6 Zn and 260.06mg kg^{-1} Pb). Due to the low biomass production of plant roots, the assessment of total metal content in roots was performed on a single composite root sample (one replication) for each treatment.

Table 2. Zn, Pb and Cd concentration (mg kg^{-1}) in *P. halepensis* tissues before planting (T0) and after 6 months of experiment (T6); R: roots; Epi: Epigean parts; <dL: lower than the detection limit; for epigean parts mean ± SD, n=3

Metals	Parameters		**NOC**	**CP**	**CPC**	**S**
Zn	Zn-R (mg kg^{-1})	T0	52.7	52.7	52.7	7459.5
		T6	45.6	6254.7	534.7	10768.5
	Zn-Epi (mg kg^{-1})	T0	0.6±0.1	0.6±0.1	0.6±0.1	701.8
		T6	0.9±0.4	263.1±56.5	24.2±4.2	505.6±27.7
Pb	Pb-R (mg kg^{-1})	T0	5.7	5.7	5.7	6885.7
		T6	12.06	5342.4	707.3	11427.2
	Pb-Epi (mg kg^{-1})	T0	<dL	<dL	<dL	535.2
		T6	<dL	173.1±53.5	21.9±3.4	260.06±21.2
Cd	Cd-R (mg kg^{-1})	T0	<dL	<dL	<dL	95.05
		T6	<dL	44.7	4.6	97.7
	Cd-Epi (mg kg^{-1})	T0	<dL	<dL	<dL	7.3
		T6	<dL	1.6±0.4	<dL	6.4±0.3

Plant Biometric Parameters: Survival, Elongation and Biomass Growth

Figure 3 shows the assessment of biometric parameters measured for *P. halepensis* in terms of plant survival (at T6) as well as root and epigean elongation and biomass production at the beginning (T0) and at the end (T6) of phytoremediation experiment for each treatment. The highest plant survival percentage was reported in not-contaminated substrate (NOC) and in S (upto 100%) followed by CP (93.3%) at T6. Further, the plants grown in CPC substrate showed 60% of survival rate (Figure 3, row1). ANOVA shows no statistically significant differences (p<0.05) among treatments (Figure 3a, row2) in terms of root and epigean elongation, except for plants that grew on the S substrate which were significantly lower compared to the other treatments. Significant differences ($p<0.05$) were assessed in the epigean elongation during the time (Figure 3b, row2) in all treatments, whilst root elongation varied significantly over time only in CP and S substrates. The epigean biomass production in substrate S was significantly lower than that observed in other treatments, whereas the root biomass production was similar in NOC, CPC, S and significantly higher in CP (Figure 3a, row3). During the time, the epigean biomass significantly grew in all treatments, whilst roots biomass showed no significant changes ($p<0.05$) after 6 months of experiment in CPC and NOC (Figure 3b, row3).

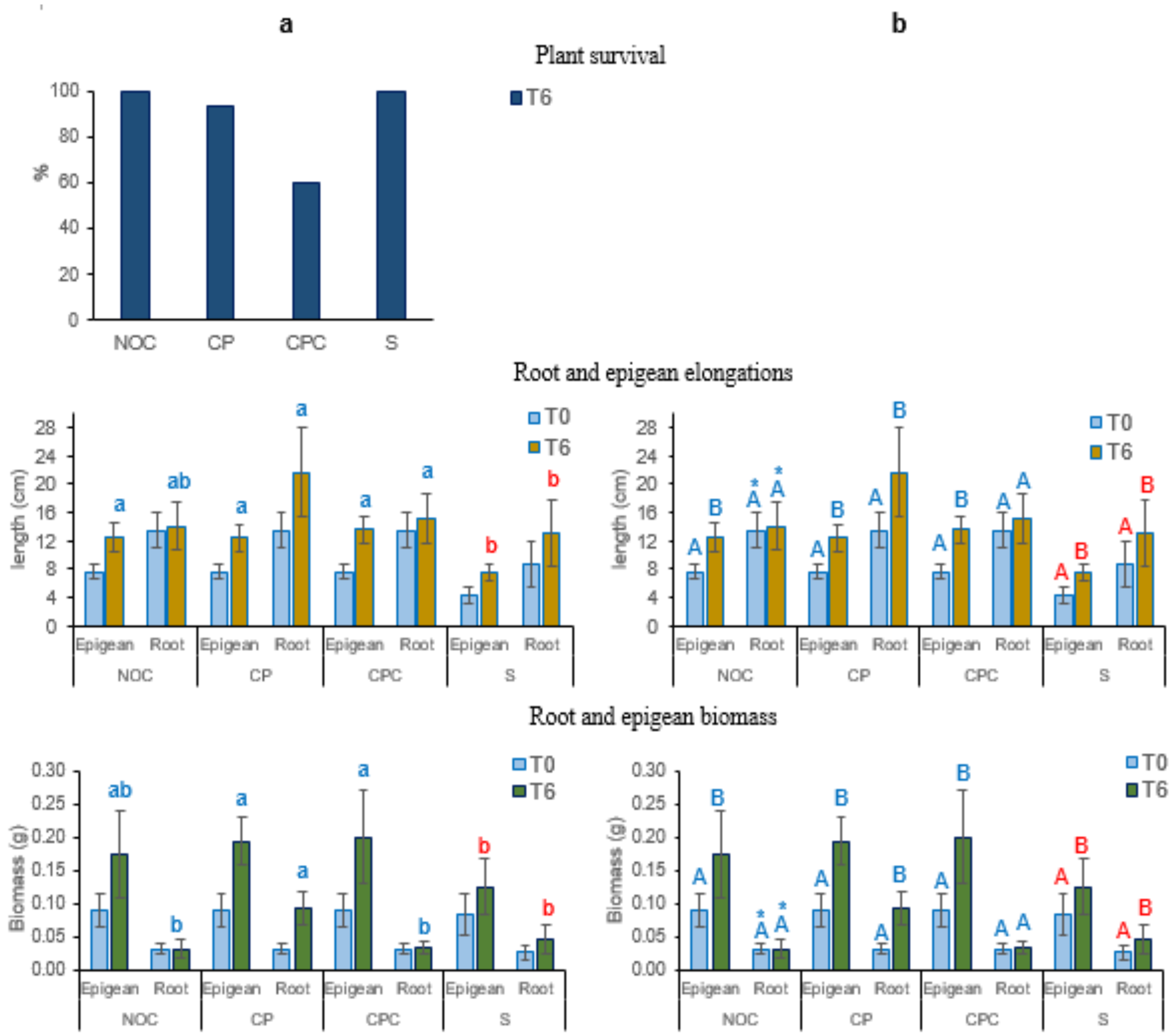

Figure 3. Biometric parameters of *P. halepensis* during the trial (mean values± SD; n=15) and statistical analysis: (**a**) statistical analysis of biometric parameters among treatments at a fixed time (T6); (**b**) statistical analysis of biometric parameters in each treatment over time (from T0 to T6); different letters indicate statistical significant differences at p<0.05; different colors of letters indicate plant references (blue color for plant obtained from nursery and red for the plant collected from CP site)

Figure 4 summarized the variation of biometric parameters calculated in T6 for all studied treatments in comparison with the control sample (T0). It shows that plant survival was very high in all substrates, with exception of the compost-amended substrate (60%). The root length and biomass increased over time, especially in CP and S, the most contaminated substrates, whilst this increase was reduced in CPC and even more in the reference treatment. The epigean length and biomass show similar increases among treatment substrates.

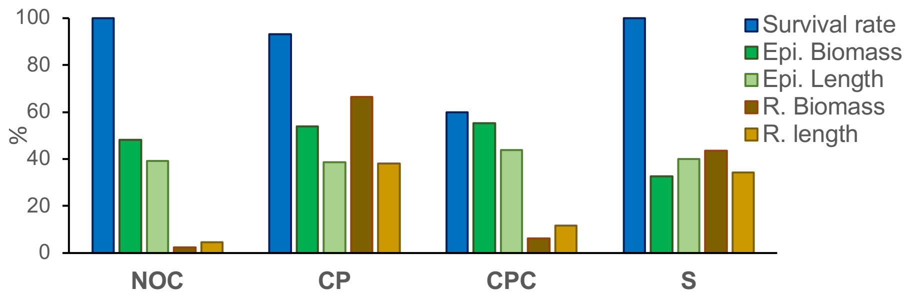

Figure 4. Variation of biometric parameters calculated in all treatments at the end of phytoremediation experiment (six months); R: root; Epi: epigean

Biological Accumulation Parameters and Phytoremediation Potential

The biological accumulation parameters (BCF, BAC and TF) were calculated for root and epigean samples through the phytoremediation test in order to evaluate the phytoremediation potential of *P. halepensis* (Figure 5). In all substrates, the measured values of investigated metals are consistently very low (<1). In detail, the BCF values of CPC for all the metals uptake from soil to roots are significantly very lower than those of CP and S. In not-contaminated substrate (NOC), the BCF values for Zn and Pb were higher (0.8 Zn and 0.6 Pb) than those of CP and CPC samples. The BAC values for Zn and Pb were generally very similar for all substrates (0.01–0.03), except in CPC which shows the lowest BAC for all metals. The BAC values related to the uptake of Cd in S and CP substrates were higher than in not-contaminated and compost-amended ones. The translocation of Zn, Pb and Cd to the epigean organs was generally low (TF<1) in all substrates. Higher TF values for Cd have been measured in S (0.06) and CP (0.04). However, the TF of Zn and Pb were the same in all measured substrates. The lowest BAC and BCF value for all metals were measured in CPC sample (Figure 5).

Table 3 shows the Pearson correlation between the total Zn, Pb, and Cd content in the substrates, the content in P. halepensis root and epigean samples, and the concentrations of DTPA-extracted metals at the end of the six-month trial (T6). The total Zn and Pb contents in substrates are strongly positively correlated with their concentration in roots and epigean samples of P. halepensis. The matrix shows that the bioavailability of all metals is positively correlated with their concentrations in P. halepensis roots and epigean samples. Moreover, the bioavailability of all metals is positively correlated with the total Zn content, while there was no reported positive correlation for total Pb and Cd content in substrates.

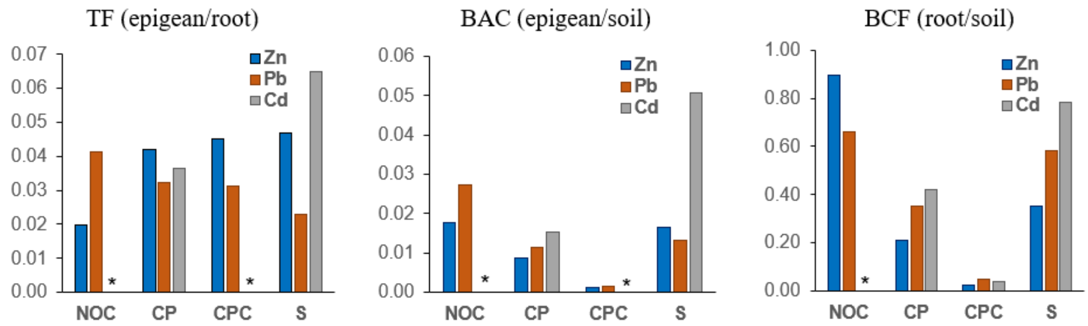

Figure 5. Biological accumulation indexes (BAC, TF and BCF) through the phytoremediation test (T6) and calculated from the data in table 1 and Table 2; * indicated that the calculated values for Cd were lower than detection limit in some substrates (<dL)

Table 3. Correlation matrix between the total metals contents in soil (T), metals contents in Pinus halepensis root (R), epigean organs (Epi), and the bioavailable contents of Zn, Pb and Cd (bf) at the end of the six months study; correlation (r > 0.7) is significant at the 0.05 level (2-tailed).

Samples	T- Zn	T- Pb	T- Cd	Zn-R	Pb-R	Cd-R	Zn-Epi	Pb-Epi	Cd-Epi	Zn-bf	Pb-bf	Cd-bf
T-Zn	1	0.96	0.94	0.80	0.78	0.77	0.73	0.77	-	0.79	0.80	0.82
T-Pb	0.96	1	0.97	0.80	0.80	0.79	0.70	0.70	-	-	-	0.71
T-Cd	0.94	0.97	1	-	-	-	-	-	-	-	-	-
Zn-R	0.80	0.80	-	1	0.99	0.99	0.99	0.99	0.96	0.94	0.93	0.95
Pb-R	0.78	0.80	-	0.92	1	1	0.99	0.99	0.98	0.89	0.88	0.90
Cd-R	0.77	0.79	-	0.92	1	1	0.99	0.99	0.98	0.88	0.88	0.90
Zn-Epi	0.73	0.70	-	0.99	0.99	0.99	1	0.98	0.96	0.90	0.89	0.91
Pb-Epi	0.77	0.70	-	0.99	0.99	0.99	0.98	1	0.90	0.93	0.93	0.94
Cd-Epi	-	-	-	0.96	0.98	0.98	0.96	0.90	1	0.77	0.76	0.78
Zn-bf	0.79	-	-	0.94	0.89	0.88	0.90	0.93	0.77	1	1	0.99
Pb-bf	0.80	-	-	0.93	0.88	0.88	0.89	0.93	0.76	1	1	0.99
Cd-bf	0.82	0.71	-	0.95	0.90	0.90	0.91	0.94	0.78	0.99	0.99	1

Discussion

The results showed that Zn, Pb and Cd concentrations measured in contaminated (CP and S) and compost-amended (CPC) substrates were above the maximum threshold contamination levels estimated by Italian law for the industrial purpose (1500 Zn, 1000 Pb and 15mg kg^{-1} Cd; Decreto Legislativo n.152. 2006, Guri 2006) with the exception of Cd in CPC substrate that was below the threshold limit.

It should be considered that Zn is an essential element for plant growth, whereas Pb and Cd are highly toxic for plants (Nagajyoti et al. 2010; Kabata-Pendias 2011). Further, all metals concentration measured in root and epigean organs of *P. halepensis* grown in CP and S samples were above the reported phytotoxic levels for plants (20–100 Zn, 10–20 Pb and 5–10mg kg^{-1} Cd; Rathore et al. 2019) at the end of the trial, except for Cd in epigean organs. This indicates the *P. halepensis* tolerance and adaptability to grow and its capacity to develop the mechanisms to survive in the contaminated mine waste. All metal concentrations measured in roots were higher than epigean organs of *P. halepensis*, indicating metal tolerance and exclusion mechanisms of plant for these metals. These outcomes are in agreement with the previous studies carried out by Disante et al. (2010) reporting high Zn accumulation in roots than in the aerial parts of *P. halepensis*. The effect of organic-amendments to reduce the metal uptake in roots are reported for some plant species growing in similar polluted mine sites (Bacchetta et al. 2012, 2015; Conesa and Párraga-Aguado 2019; Boi et al. 2021). Likewise, the finding of this study also reveals that the metal content (total metal and bioavailable fractions) and the metal uptake into the plant tissues were generally lower in the presence of compost (30% w/w) in CPC than in CP and S substrates (see Table 1). Indeed, all metals accumulation in epigean organs and Cd in roots of *P. halepensis* grown in CPC were below the phytotoxic levels for plant at the end of six-months study (T6). Thus, compost addition can have a positive effect on the reduction of Zn, Pb and Cd bioavailability in soil and their concentration in plant tissues over time (see Table 2).

The calculated variation of biometric parameters in T6 shows that plant survival was higher in contaminated substrates. This finding shows the higher adaptability of *P. halepensis* to highly contaminated mine waste than other native plants species (i.e., *H. microphyllum* subsp. t*yrrhenicum*, *P. lentiscus* and *P. australis*) that were investigated in different phytoremediation experiments in the same mine tailing (Bacchetta et al. 2015; Boi et al. 2021). This confirms the great potential of *P. halepensis* to tolerate polluted substrates and matrix toxicity, even though, the visual symptoms of metal toxicity, such as chlorosis were distinguished in the needles of seedlings grown in CP substrate at the end of trial. Moreover, the exposure of young seedlings to the stress conditions after the addition of compost induced negative effect on plant survival in compost-amended substrates (CPC) over time. The significant increase of plant growth (roots elongation and biomass) in both CP and S substrates and similar growth of epigean parts in all substrates reveal that compost addition seems not to influence the survival rate nor the elongation and biomass production of root and epigean organ in *P. halepensis*. However, compost could improve the physico-chemical properties of the contaminated substrate by slightly increasing organic carbon, total nitrogen and electrical conductivity and decreasing pH value to slightly acidic conditions (see Table 1). This is in agreement with the findings of Concas et al. (2006), which reveal that organic-amendments could be able to improve the physical properties of mine tailings in terms of water retention and bulk density. Other studies have also suggested that soil organic-amendments can decrease metal availability by the formation of organic metallic complexes (Clemente et al. 2006; Rodríguez et al. 2016). Moreover, the CP and S substrates showed lower organic-carbon contents than CPC, indicating the presence of inorganic carbon in the form of carbonate (calcite and dolomite) which is consistent with the carbonate lithology rich in alkaline waste materials in CP mine area (Bechstädt et al. 1994; Bacchetta et al. 2015).

Further, the addition of compost, especially at the first stage of plant growth, and the exposure of young seedlings to stress conditions on planting in contaminated matrices induced increasing plant mortality in a short time (Disante et al. 2010). This may explain the low elongation and biomass production in root samples in compost-amended substrate (CPC). Thus, further investigation on the long-term application of amendment is advisable to better understand the effectiveness of compost on the growth of *P. halepensis* root samples over long-time.

The analysis of PCA confirmed that both CP and S substrates are similar and statistically correlated with Zn, Pb and Cd concentration in their substrates as well as in *P. halepensis* tissues, considering their differences with compost-amended substrate. Moreover, the biological accumulation parameters were generally very low (BAC, BCF and TF <1) in all substrates, indicating the phytostabilization/revegetation potential of *P. halepensis* that can restrict the translocation of Zn, Pb and Cd from soil to roots and more specifically to the aerial parts of plant. Thus, due to the low capability to accumulate Zn, Pb and Cd into epigean organs, and according to what has already been reported in the *in-situ* investigation carried out by Kharazian et al. (2022a), *P. halepensis* with low translocation factor (TF< 1) can be considered as a pioneer species suitable for revegetation in highly polluted mine waste materials.

Conclusion

This study was carried out to evaluate the phytoremediation potential of *P. halepensis* growing on highly contaminated mine waste to provide information on potential revegetation and recovery of mine tailing. The results reveal that *P. halepensis* can possess suitable characteristics for phytostabilization projects: (i) the high concentrations of Zn, Pb, and Cd in contaminated substrates and in roots samples, as well as the overall plant survival and growth data in short-term highlighted the tolerance and adaptability of the plant to survive and grow in contaminated mine substrates; (ii) the metals bioavailability and the low BAC, BCF, and TF values (<1) emphasize the phytostabilization potential of *P. halepensis*; (iii) the addition of compost-amendment had a negative influence on plant survival and growth, however, it improved the organic-carbon and nitrogen contents in the substrate and mitigated the heavy metals toxicity in the plant through decreasing total metal content in the soil, the low bioavailable fraction and metal accumulation in roots and epigean organs of *P. halepensis*. Thus, compost can be advisable in phytostabilization projects considering further in-situ investigations applying different compost qualities/quantities to provide better insight into *P. halepensis* physiological adaptation in the mine tailing site.

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