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Evaluating Poplar Hybrids and Companion Herbaceous Species for Phytoremediation of Riotinto Mine Soils: Survival and Trace Element Accumulation Assessment

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Ecological restoration of mine dumps poses a significant global challenge. This study explores the viability of plant growth in the soils of the Riotinto mine, where high concentration of trace elements hinders vegetation establishment. Greenhouse experiments were conducted using 5 poplar hybrids (PA148, PA149, PA152, PA153 and the parental P10) and two herbaceous species, *Brassica juncea* L. and *Lablab purpureus*, grown in both contaminated and uncontaminated soils. The contaminated soil (RT) had an initial acidic pH of 3.07, which required the addition of a sugar lime amendment (SL), to reduce the availability of cationic trace elements. Poplar hybrids were grown for 3 months in both uncontaminated (C) and amended (RT + A) soils, while the herbaceous species were grown for 1 month in C, RT, and RT + A soils. Soil parameters, including pH, organic matter (OM), and both pseudototal and available trace element concentrations (extracted using 0.01 M $CaCl_2$), were analyzed. Plant biomass, trace element accumulation, and nutritional content in aerial tissues were also assessed. All poplar hybrids survived, with P152 and P153 demonstrating the highest survival rates and significant accumulation of trace elements, particularly Cd, Pb and Zn, in their leaves under RT + A soil conditions. *B. juncea* showed very limited growth compared to the other species, although it accumulated the highest concentration of trace elements. In contrast, *L. purpureus* demonstrated successful germination and growth in RT soil, showing strong tolerance to both acidity and contamination while maintaining the lowest levels of trace elements in its aerial tissues. These results highlight the importance of selecting appropriate plant species and soil amendments tailored to site-specific conditions and remediation goals.

Keywords: heavy metals, *Brassica juncea*, *Lablab purpureus*, populus, amendments

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INTRODUCTION

Mining activities drastically alter the surrounding landscape, destroying vegetation, soil, geological formations, and topography (Wang et al., 2014). Historically, large volumes of waste generated during mining were discarded at mine dumps without adequate treatment to remove toxic trace elements, leading to widespread environmental contamination (Willscher et al., 2010). Mine dumps are characterized by poor texture, low soil content, high porosity, and minimal organic matter, all of which severely limit plant growth (Pashkevich, 2017). The high levels of trace elements commonly found in mine dumps exacerbate contamination problems, making ecological rehabilitation efforts more complex (Banerjee et al., 2019). Consequently, restoring these degraded sites constitutes a critical challenge in environmental management worldwide.

Among the biological approaches to remediate contaminated soils, phytoremediation has emerged as a particularly important strategy in recent decades, owing to its cost-effectiveness, relatively low technical demands, and applicability for *in situ* treatment. Furthermore, it contributes to mitigating soil erosion and controlling trace element pollution, making it especially valuable for the restoration of mine dump sites (Zhou et al., 2021). Additionally, it stands out as an environmentally friendly technique that promotes the growth of vegetation in contaminated areas, contributing to ecological restoration (Yadav et al., 2018). A significant challenge for phytoremediation technologies is dealing with the variety of trace elements found in contaminated soils. While some plant species exhibit high tolerance to specific contaminants, the variety and complexity of contaminated soils can hinder crop establishment and effective phytoremediation. One promising strategy is intercropping—cultivating multiple plant species that provide distinct and potentially complementary ecosystem services (Brereton et al., 2020). Indeed, Desjardins et al. (2018) concluded that employing multiple species could be a broadly applicable and preferable approach for phytomanagement of complex, real-world contaminated sites.

Poplar trees (*Populus* sp.) are widely utilized in phytoremediation due to their adaptability and effectiveness in extracting and stabilizing contaminants from soil (Ciadamidaro et al., 2024). These trees have an extensive root system, fast growth, substantial biomass, a strong ability to accumulate trace elements, and high stress tolerance, making them valuable candidates for remediating soils contaminated with trace elements (Hao et al., 2020). Additionally, their stems exhibit a remarkable ability to accumulate substantial amounts of trace elements, thereby contributing to the reduction of soil toxicity. For phytoremediation, hybridization of *Populus* sp. is carried out both between and within species, as well as across various sections of the genus, to produce diverse hybrid varieties. This approach aims to enhance trace element accumulation and stress tolerance, enabling the development of poplar hybrids with desirable traits for phytoremediation and optimal growth performance (Li et al., 2024). Selecting specific hybrids with high tolerance to trace elements and strong adaptability to adverse conditions is critical for advancing soil remediation efforts in practical field applications (Suo et al., 2021).

This study examines one soil of the Riotinto mine, where the extreme environmental conditions pose significant challenges to vegetation establishment, thereby hindering efforts to enhance ecosystem health. Therefore, testing plant growth under controlled conditions is necessary before conducting experiments in real-field settings in these soils. This research focuses on a greenhouse experiment aimed at evaluating the feasibility of using poplars in combination with two companion herbaceous species, *Brassica juncea* L. and *Lablab purpureus* L., as an effective strategy for soil remediation. The main objective was to identify which poplar hybrid performs best in contaminated soils, evaluating both survival rates and the accumulation of trace elements in the aerial parts, with the aim of applying the findings under real field conditions. Additionally, the study evaluates the growth performance of two selected herbaceous companion species based on their biomass production. We hypothesize that poplar hybrids (*Populus* spp.), *B. juncea*, and *L. purpureus* can successfully establish and grow in contaminated soils from the Riotinto mine. While this study evaluates each species independently, the broader objective is to identify suitable candidates for future intercropping systems.

MATERIALS AND METHODS

Study Area

The Ríotinto mining district, located within the Iberian Pyrite Belt, is one of the world's largest polymetallic mining areas, with a rich history dating back to Roman times. Extensive mining activities have significantly impacted the environment, including landscape alteration, deforestation, and pollution of soil, rivers, and air. This is largely due to open-pit mining, timber use in smelting, acid rain from sulfur emissions, and contamination from mine waste dumps exposed to erosion. The surrounding soils, within a 5–7 km radius, contain elevated concentrations of potentially toxic elements (**Table 1**) (Vázquez-Arias et al., 2023).

Currently, the mine is operated as an open-pit copper mine by Atalaya Mining, processing 15.5 million tons of ore annually with a copper grade of 0.37% and a cut-off grade of 0.14%. The site covers 35 km^2 and is divided into three main areas: the southern sector (with benches and waste dumps), the central sector (housing processing facilities), and the northern sector (tailings ponds and water management). The tailings ponds—Gossan, Cobre and Aguzadera—are situated within the Odiel river basin (Diaz et al., 2024).

Experimental Design and Monitoring for Hybrids

Soil was collected from the site designated for a future field experiment (37°41′26.4″N, 6°34′01.8″W), located within the Riotinto mining area. To obtain a representative sample of the site, systematic sampling was conducted across an area of approximately 600 m^2 in collaboration with Atalaya Minning.

TABLE 1 | Concentration of pseudototal trace elements (mean ± sd) in the contaminated soil (RT) (n = 3).

	Mean ± SD	Minimum	Maximum
As (g kg^{-1})	1.39 ± 0.68	0.84	2.53
Cd (mg kg^{-1})	1.55 ± 0.95	0.50	2.72
Cu (g kg^{-1})	1.13 ± 0.63	0.62	2.13
Fe (g kg^{-1})	269 ± 140	176	507
Hg (mg kg^{-1})	7.08 ± 5.88	1.79	16.3
Mn (mg kg^{-1})	199 ± 188	40.8	523
Ni (mg kg^{-1})	8.62 ± 2.95	4.13	11.7
Pb (g kg^{-1})	6.38 ± 3.85	3.96	13.2
S (g kg^{-1})	9.26 ± 7.75	3.71	22.8
U (mg kg^{-1})	0.56 ± 0.17	0.27	0.74
Zn (g kg^{-1})	0.36 ± 0.13	0.19	0.49

Fifteen evenly distributed sampling points were selected across the site. At each point, around 10 kg of soil were extracted from the top 0–20 cm of the soil profile using a shovel. The collected soil was placed in individual sacks, transported to the greenhouse, and air-dried. To minimize spatial variability, the contents of the sacks were thoroughly mixed and homogenized before being used in the experiment.

The experiment took place at the experimental farm "La Hampa" (Coria del Río), part of the IRNAS-CSIC research facilities. A total of 65 pots (2 L capacity each) were prepared: 50 were filled with soil from Riotinto (RT) and 15 with control soil (C) sourced from agricultural land on the same farm. The RT and C soils differ markedly in their physicochemical characteristics. The RT soil is highly acidic, with a pH of 3.07 (in KCl) and 3.28 (in water), whereas the C soil is alkaline with a pH of 7.91 (in water), indicating contrasting acidity–alkalinity regimes. Electrical conductivity (EC) is substantially higher in RT (0.896 dS m^{-1}) compared to C (0.231 dS m^{-1}), reflecting a greater concentration of soluble salts in the contaminated soil. Organic matter (OM) content is notably depleted in RT (0.30%) relative to C (2.54%), suggesting poor nutrient availability and limited biological activity. In terms of texture, both soils are classified as sandy loam.

Given the strongly acidic nature of the contaminated RT soils, a sugar lime amendment (SL) was incorporated at a rate of 30 t ha^{-1} (RT + A) in the first 10 cm of the soil of the pots. Sugar lime, which contains 70%–80% calcium carbonate ($CaCO_3$), effectively neutralizes soil acidity through chemical buffering reactions, gradually raising the soil pH from highly acidic to near-neutral levels (Madejón et al., 2010). This amendment was necessary to improve the soil chemical properties and create favorable conditions for plant establishment. Due to the high complexity, cost, and extended duration of the hybrid field experiment, applying the amendment prior to planting was essential to ensure plot viability and plant survival. A non-amended control soil was not included, as the extreme acidity of the native RT soil would have precluded plant growth and compromised the feasibility of the experimental design.

Five poplar varieties, consisting of one parental species and four hybrids, were evaluated in this study. The hybrids were supplied by Phytowelt (Germany), an industrial partner in the European EDAPHOS project. Hybrid P10 was derived from a cross between *Populus nigra*—recognized for its high wood quality—and *Populus maximowiczii*, known for its rapid growth rate. The additional hybrids, P148, P149, P152, and P153, were generated from backcrossing P10 with *Populus alba*. The latter species is characterized by drought and pathogen resistance and its ability to thrive in nutrient-poor soils, although the hybrids used in this study have not been previously assessed for growth in contaminated environments.

In early November 2023, uniform cuttings (25–30 cm in length) containing only buds (no leaves) were planted for each hybrid. A total of 13 cuttings per hybrid were established, with 10 planted in contaminated Riotinto soil and 3 in uncontaminated control soil. After planting, sprinkler irrigation was scheduled for 3 min, three times per week, to maintain adequate moisture.

Physiological monitoring began 8 weeks post-planting, coinciding with leaf emergence. Every 2 weeks, leaf chlorophyll content was assessed on all five poplar varieties using a SPAD-502 chlorophyll meter (Konica Minolta, Japan). For each plant, three measurements were taken from comparable leaves and averaged to obtain individual chlorophyll values (Chang and Robinson, 2003). The plants were maintained under these conditions for a total of 3 months.

Experimental Design for Companion Herbaceous Species

The experiment was carried out in a controlled-environment growth chamber at IRNAS-CSIC research facilities. Environmental conditions were maintained with a 16-h photoperiod (light from 07:00 to 23:00) and a constant temperature of 24 °C. A total of 24 pots (1 L each) were prepared for each plant species, with eight pots assigned to each soil type (RT, RT + A, and C), consistent with the hybrid poplar experiment setup.

To evaluate the performance of the companion herbaceous species under highly acidic conditions, seeds were also sown in untreated RT soil without amendment. For *B. juncea*, 1 g of seeds was evenly distributed per pot, whereas for *L. purpureus,* four seeds were planted per pot. Plants were irrigated every 2 days to maintain optimal soil moisture and grown for 1 month.

Physiological measurements were performed during the third and fourth weeks of growth. Leaf chlorophyll content was assessed in *L. purpureus* using a SPAD-502 chlorophyll meter (Konica Minolta, Japan), following the same protocol used in the poplar hybrid experiment. Chlorophyll measurements could not be obtained for *B. juncea* due to its limited leaf size at this stage.

After 1 month of growth, the entire aerial biomass from each pot was harvested. Fresh biomass was recorded immediately, followed by oven-drying at 70 °C for 48 h to determine dry biomass. The dried plant material was subsequently analyzed for nutrient and trace element concentrations.

Soil and Plant Analyses

Soil samples were collected both at the start and end of the experiment. Samples were air-dried at 40 °C, sieved to <2 mm, and analyzed for chemical properties. Soil pH was measured in a

1 M KCl extract (1:2.5, m/v) using a CRISON micro pH 2002 m, and electrical conductivity (EC) was determined in a 1:5 soil-to-water extract. Total organic carbon (TOC) content was quantified using a ThermoFlash 2000 NC analyzer. For trace element determination, soil was finely ground and sieved to <60 μm. Pseudototal trace element concentrations were determined via aqua regia digestion (HNO_3:HCl, 1:3) using a Digiprep MS block digester (SPS Science), while bioavailable fractions were estimated through extraction with 0.01 M $CaCl_2$ (1:10, m/v) following Houba et al. (2000). Trace elements in all the extracts were determined by ICP-MS (Inductively Coupled Plasma Mass Spectrometry (Agilent 7800). For plant analyses, only leaves of the poplars were sampled to determine nutrient and trace element concentrations. Due to the limited vegetative growth observed, all leaves from each pot were harvested at the end of the 3-month period to ensure adequate biomass for chemical analysis. For each hybrid and soil type, leaves from 3 to 5 pots (depending on the available biomass) were combined to create a composite sample, resulting in three replicates per hybrid and soil. In the case of *B. juncea* and *L., purpureus* for the RT + A and RT soils (RT only for Lablab), two to three pots per soil type were selected—depending on biomass availability—to prepare composite samples, also resulting in three replicates per treatment.

Vegetal samples were rinsed in a 0.1 N HCl solution for 15 s, followed by a rinse in distilled water for 10 s, and then oven-dried at 70 °C. Dried plant material was ground and sieved through a 500-μm stainless-steel sieve before analysis preparation.

Trace elements in plant tissues were determined using ICP-MS after wet digestion with concentrated HNO_3 in a Digiprep MS block digester. Analytical quality was ensured using reference materials, with ERM CC141 for soil and INCT-OBTL-5 (tobacco leaves) for plants, achieving recovery rates between 90% and 105%. All trace elements and nutrients concentrations were expressed on dry weight.

Statistical Analysis

For data analyses, the IBM SPSS Statistics 29.0 software was used. Analysis of variance (one-way ANOVA) and Tukey HDS post-hoc tests were conducted to evaluate the effect of different soil on the pH, TOC, psuedototal and available trace elements in soils and plants. Previously, the data were subjected to the Kolmogorov-Smirnov test to check for normal homogeneity of variances. If the distribution was not normal, a logarithmic transformation was applied, and the normality test was performed again. If the Kolmogorov-Smirnov test was still not satisfied, a non-parametric test was used (Mann-Whitney U for mean comparisons and Kruskal-Wallis for variance analysis). In all cases, a significance level of $p < 0.05$ was employed.

RESULTS

Soils

The addition of the amendment (SL) raised the soil pH by 5 units, from 3.10 to 8.15, in the top 10 cm of soil. An increase in EC was also observed after the amendment addition (from 0.896 to 1.422 dS m^{-1}). The RT soil had low soil TOC, which was improved from 0.30% to 0.46% after the SL addition. This increase in OM may also enhance the low fertility of this soil.

TABLE 2 | Chemical properties and pseudototal metal concentrations (mean ± SD) in non-contaminated control (C) and amended contaminated soil (RT + A).

	C	RT + A
pH	8.36 ± 0.06	8.15 ± 0.01
EC (dS m^{-1})	0.20 ± 0.01	1.42 ± 0.13
TOC (%)	1.92 ± 0.10	0.46 ± 0.04
As (mg kg^{-1})	4.85 ± 0.84	829 ± 17.5
Cd (mg kg^{-1})	0.06 ± 0.00	2.13 ± 0.07
Cu (mg kg^{-1})	60.8 ± 2.84	2,167 ± 80.0
Fe (g kg^{-1})	10.5 ± 1.60	406 ± 19.6
Hg (mg kg^{-1})	0.02 ± 0.004	8.39 ± 0.6
Mn (mg kg^{-1})	234 ± 11.6	68.5 ± 6.20
Ni (mg kg^{-1})	8.83 ± 0.52	5.26 ± 0.57
Pb (mg kg^{-1})	23.0 ± 17.4	11,949 ± 427
S (mg kg^{-1})	296 ± 46.1	11,409 ± 297
U (mg kg^{-1})	0.26 ± 0.02	0.61 ± 0.06
Zn (mg kg^{-1})	24.1 ± 1.34	338 ± 16.6

EC: electrical conductivity.

TABLE 3 | pH and available trace elements (extracted with 0.01 M $CaCl_2$) in non-contaminated (C), amended contaminated soil (RT + A) and unamended contaminated soil (RT) before the experiment. For each element different letters indicate significant differences among soils (p < 0.05).

	C	RT + A	RT
pH	8.01 ± 0.21 b	8.32 ± 0.19 c	3.28 ± 0.05 a
As (mg kg^{-1})	0.04 ± 0.01 a	0.30 ± 0.10 b	0.04 ± 0.001 a
Cd (mg kg^{-1})	0.0003 ± 0.0002 a	0.0008 ± 0.0003 b	0.13 ± 0.005 c
Cu (mg kg^{-1})	0.35 ± 0.10 b	0.18 ± 0.08 a	17.5 ± 0.04 c
Pb (mg kg^{-1})	0.004 ± 0.005 a	0.40 ± 0.12 b	1.87 ± 0.30 c
Zn (mg kg^{-1})	0.07 ± 0.03 a	0.08 ± 0.06 a	36.4 ± 0.45 b

At the beginning of the experiment, the pseudototal concentrations of trace elements were measured in both the C and RT + A soils. The analysis revealed significantly higher concentrations of all examined trace elements in the RT + A soils. Among these, Pb exhibited the greatest increase relative to the control, followed by As, Cd, and Cu, while Zn showed the smallest difference (**Table 2**). Notably, the addition of the amendment did not affect the total metal content in the soil (data not shown).

The area of the contaminated soils corresponds to the Iberian Pyrite Belt, specifically the South-Portuguese zone (Galán et al., 2008). The geochemical background values on this zone are 47.5, 46.4, 143 and 89 mg kg^{-1} for As, Cu, Pb and Zn. The RT soils far exceed these values, with Pb concentrations 77 times higher than background levels, Cu 45 times higher, and As 20 times higher.

Trace element availability was assessed in C, amended RT + A and unamended RT soils before the experiments (**Table 3**). As expected for mine-impacted soils, $CaCl_2$-extractable concentrations were much lower than the pseudototal values. Notably, Pb and especially As exhibited extremely low availability, with concentrations below 0.01% of their

TABLE 4 | Percentage of the hybrids poplars producing green leaves (%) in no-contaminated (C) and amended contaminated soil (RT + A).

Soil type	P148	P149	P152	P153	P10
Control	33.3	33.3	66.7	66.7	100
RT + A	10	30	80	50	60

Note: Data is based on three replicates for control soil and ten replicates for contaminated soil.

pseudototal content. In contrast, Cd and Zn displayed higher availability, reaching approximately 10% of their pseudototal concentration.

A key difference between RT and RT + A soils, was the previously mentioned variation in pH values. This resulted in a drastic and significant reduction in the availability of cationic trace elements (Cd, Cu, Pb, and Zn) in the RT + A soil. However, in the case of As, which exists predominantly as an anion, the pH increase resulted in a substantial rise in its availability, with concentrations increasing from 0.004 mg kg^{-1} at pH 3 in RT soils to 0.30 mg kg^{-1} in RT + A soils. It should be noted that the $CaCl_2$ extraction method has limited reliability in accurately estimating As availability. Therefore, the results obtained using this method should be interpreted with caution and considered as indicative rather than definitive.

Poplar Development and Companion Species Biomass

After 3 months of growth, poplar development was evaluated based on the proportion of cuttings producing green leaves in each soil type (**Table 4**). In the control soil, the parental hybrid P10 demonstrated the highest growth performance, with 100% of cuttings producing green leaves. In the RT + A soils, hybrid P152 showed the highest proportion of cuttings producing green leaves (80%), followed by P10 and P153. Conversely, hybrids P148 and P149 exhibited poor performance in RT + A, with only 10% and 30% of cuttings producing green leaves, respectively, indicating limited suitability for these conditions. Moreover, the low survival rates observed under no-contaminated soil suggest that additional factors may be influencing the growth limitations of these two hybrids.

Brassica juncea failed to establish in the untreated RT soil, and its biomass in the RT + A soils was significantly lower than in the control soil (1.00 ± 0.13 g vs. 2.86 ± 0.10 g dry weight; $U = 0.00$, $p < 0.001$). In contrast, *L. purpureus* successfully germinated and grew in all soil types. Due to its larger plant size compared to *B. juncea*, biomass was expressed on a per-plant basis, preventing direct species-to-species comparisons. Mean biomass values were 0.81 ± 0.01 g in C soil, 0.44 ± 0.20 g in RT + A, and 0.18 ± 0.03 g in RT. Biomass differences across the three soils were statistically significant ($F = 28.397$, $p < 0.001$).

Values of SPAD chlorophyll readings were recorded at several time points. For poplar hybrids, the figure presents data collected at 2 and 3 months after planting, while for *L. purpureus*, measurements were taken at 3 and 4 weeks of growth (**Figure 1**). SPAD measurements were not possible for *B. juncea* due to its very small leaves. No significant differences in SPAD values were detected among poplar hybrids or between soil types (control and RT + A); however, hybrids P148 and P149 could not be fully assessed because most cuttings failed to produce green leaves. In contrast, *L. purpureus* displayed significant variation in SPAD values among soil types both after 3 weeks ($F = 3.973$, $p < 0.05$) and 4 weeks ($F = 13.805$, $p < 0.001$) of growth. After 4 weeks, SPAD values indicated that control (C) and RT plants reduced their chlorophyll levels (**Figure 1**).

Trace Element Concentrations in Plants

Consistent with the soil chemical analyses, the most pronounced differences between control and RT + A soils were observed for Pb and As (**Figure 2**). Statistical analysis was conducted only for hybrids P152, P153, and P10 due to limited sample availability for P148 and P149 (single data point per condition). Significant differences in trace element accumulation were detected between C and RT + A soils for all analyzed elements in poplars. Lead and As exhibited the largest differences, mirroring their high soil concentrations. Variation among hybrids was significant only for Cd ($F = 6.102$, $p = 0.012$) and Fe ($F = 3.857$, $p = 0.045$). Overall, hybrids P152 and

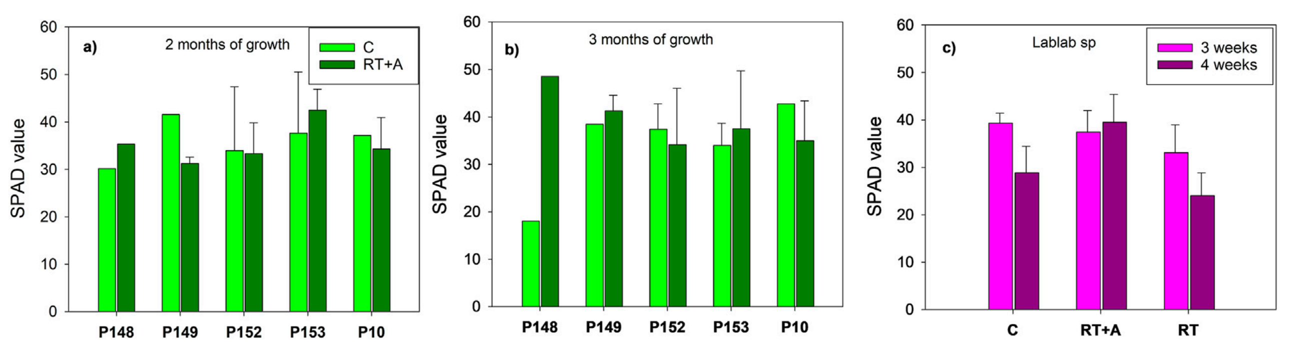

FIGURE 1 | SPAD values (mean and standard deviation) in poplar leaves at different stages of plant development: **(a)** 2 months after planting, **(b)** 3 months after planting, and **(c)** in *Lablab purpureus* 3 and 4 weeks after sowing in control (C) and contaminated amended soil (RT + A).

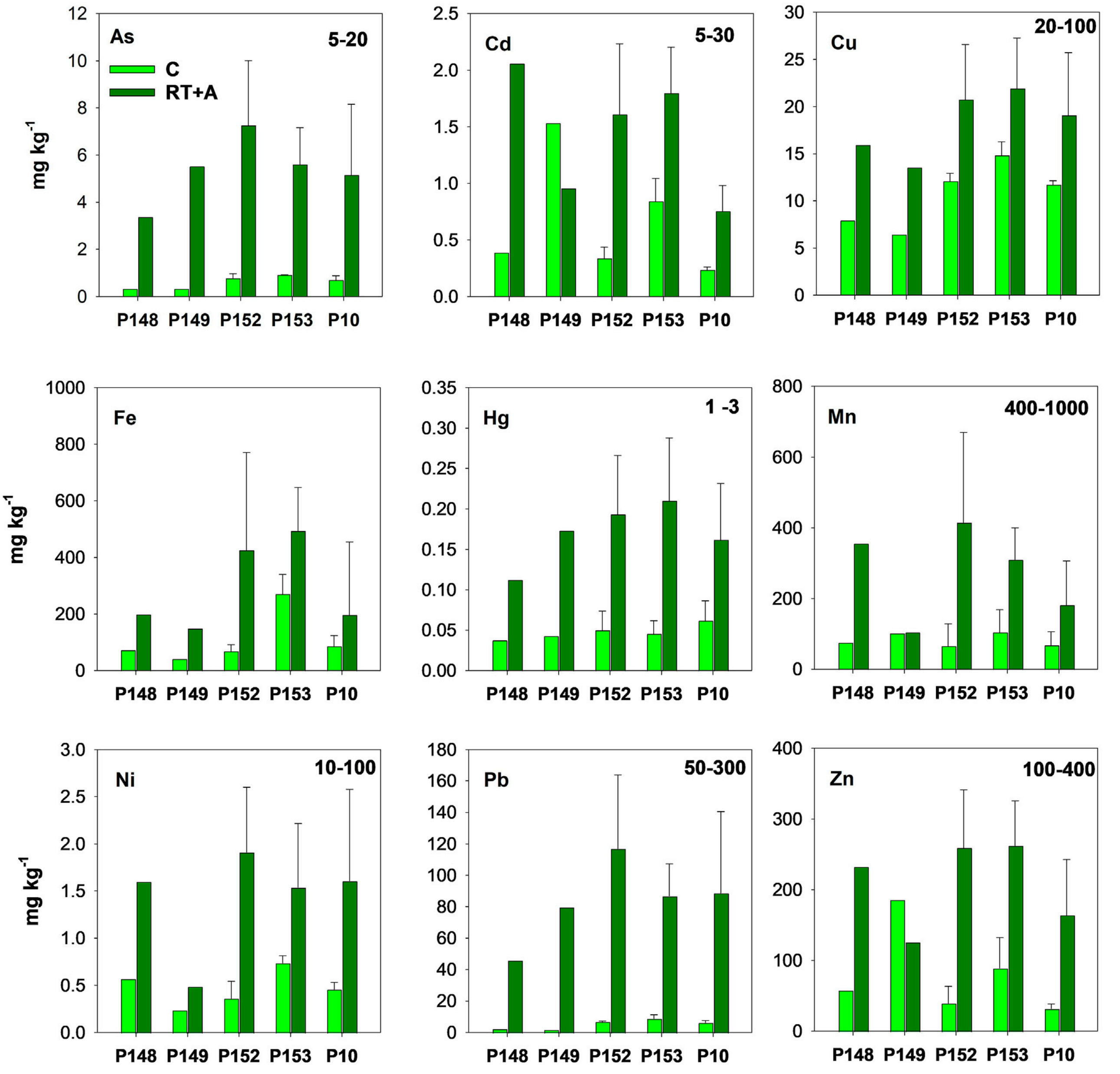

FIGURE 2 | Concentrations of trace elements (mean and standard deviation) in poplar leaves for each hybrid in control (C) and contaminated amended soil (RT + A). The range of concentrations (mg kg^{-1}) considered excessive or toxic according to Kabata-Pendias (2011) is shown in the top right corner of the figure.

P153 presented the highest concentrations of most trace elements (As, Cd, Cu, Fe, Hg, Mn, Pb, and Zn). Concentrations of As, Cd, and Pb exceeded normal leaf thresholds for all hybrids, while Mn and Zn occasionally surpassed reference levels (Kabata-Pendias, 2011). No established reference values were available for Fe.

For *B. juncea*, trace element accumulation was significantly higher in RT + A compared to C soils for all elements except Ni, which was not elevated in the soil (**Figure 3**). Arsenic levels were particularly high, and Cu, Pb, and Zn concentrations exceeded those found in poplar leaves after 4 weeks of growth.

Lablab purpureus successfully grew even in RT soils. Overall, trace element concentrations in aerial biomass were lower than those measured in *B. juncea* and poplar hybrids, despite the higher trace element availability in RT soils (**Figure 4**; **Table 3**). Except for Pb in RT, concentrations did not exceed normal plant thresholds.

Nutrient Concentrations in Plants

Macronutrient concentrations in poplar hybrid leaves are displayed in **Figure 5**. No significant differences were detected among hybrids within the same soil. Potassium showed the lowest

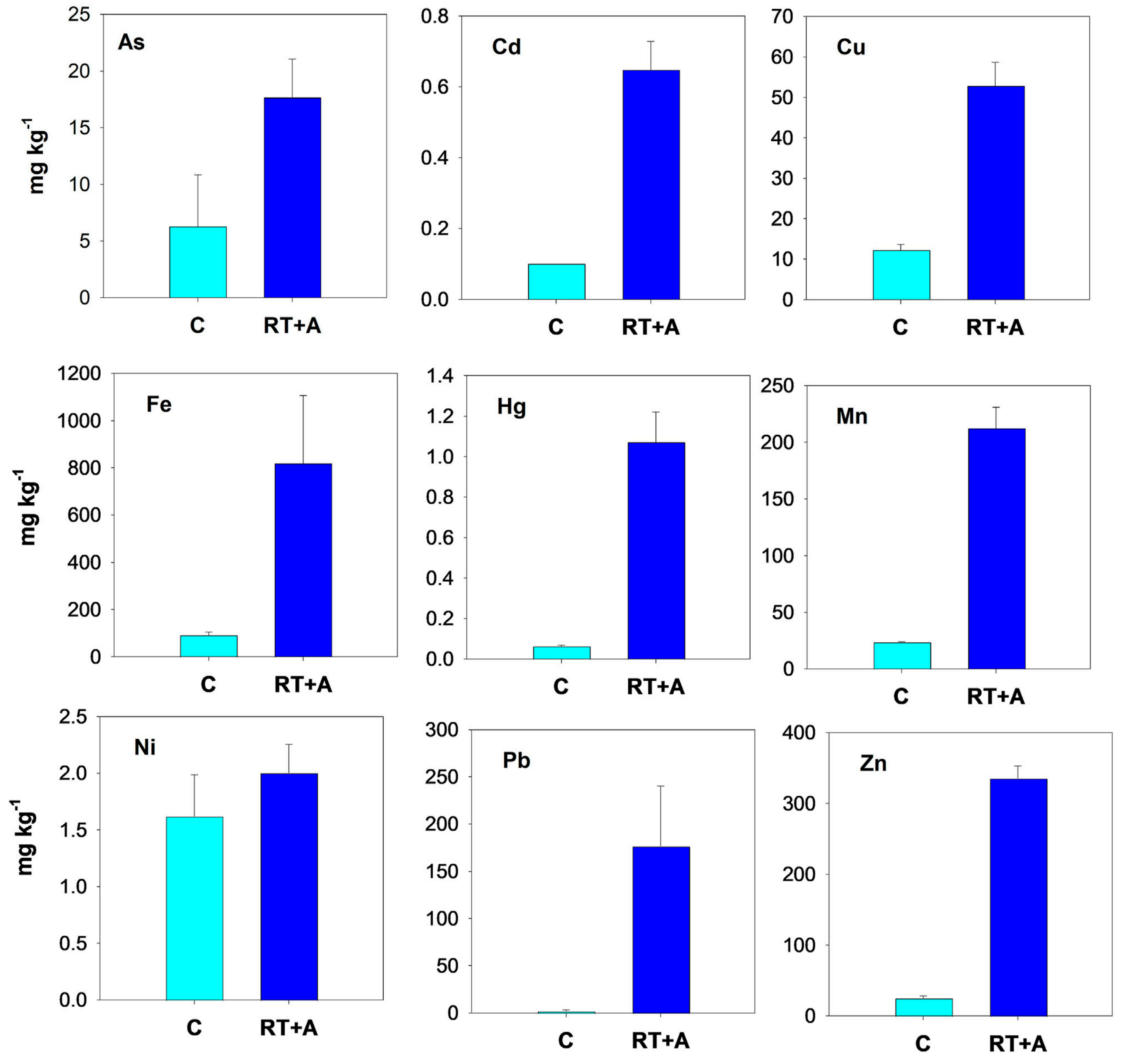

FIGURE 3 | Concentrations of trace elements (mean and standard deviation) in aerial biomass of *B. juncea* in control (C) and contaminated amended soil (RT + A).

values in RT + A leaves, with significantly higher levels in C soil for hybrids P152, P153, and P10 ($F = 49.069$, $p < 0.01$). Similar trends were observed for P148 and P149. Phosphorus concentrations were also significantly lower in RT + A soils ($F = 5.009$, $p < 0.001$), whereas Mg and S levels were significantly higher in RT + A compared to C soils ($F = 15.207$, $p < 0.001$ and $F = 87.852$, $p < 0.001$, respectively). No significant differences were found for Ca or Na.

In *B. juncea*, nutrient patterns largely mirrored those of the poplar hybrids (**Figure 6**). Ca and K levels were significantly lower in RT + A plants ($F = 107.229$, $p < 0.001$ and $F = 201.593$, $p < 0.001$, respectively), while Mg, Na, and S concentrations were significantly higher compared to C plants ($F = 202.127$, $p < 0.001$; $F = 267.457$, $p < 0.001$; $F = 508.134$, $p < 0.001$). Phosphorus contents remained similar between both soils.

For *L. purpureus*, nutrients distribution followed a pattern similar to that observed in *B. juncea* (**Figure 7**). Growth at pH 3 (RT soil) notably influenced the results. Calcium concentration was lowest in the RT soil ($H(2) = 0.027$), while K levels differed significantly across all three soils ($F = 157.314$, $p < 0.001$). Magnesium and S concentrations were significantly higher in plants grown in contaminated soils (RT and RT + A) compared to controls ($F = 29.972$, $p = 0.001$ and $F = 78.215$, $p < 0.001$, respectively). Additionally, P content was significantly greater in

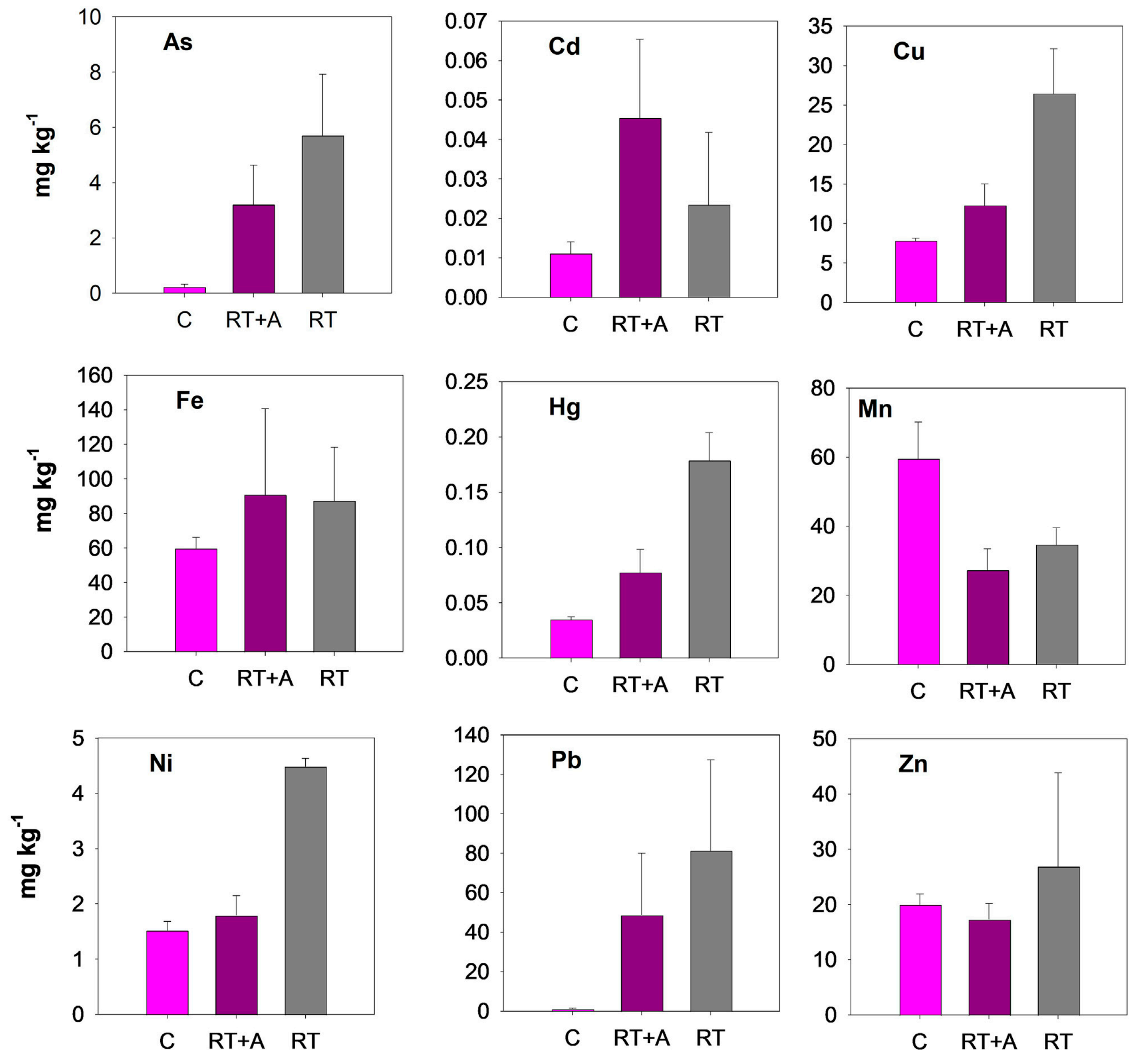

FIGURE 4 | Concentrations of trace elements (mean and standard deviation) in aerial biomass of *P. purpureus* in control (C), contaminated amended (RT + A) and in contaminated soil (RT).

RT plants than in those from the RT + A and C ($F = 66.825$, $p < 0.001$).

DISCUSSION

Soil Characteristics and Trace Element Bioavailability

The soil at the Ríotinto mine is highly acidic, which severely limits plant establishment and growth (Che et al., 2023). Previous research has demonstrated the effectiveness of soil amendments, such as SL, in ameliorating acidic conditions and promoting vegetation development. For example, Pérez de Mora et al. (2011) reported that SL application facilitated spontaneous vegetation colonization, while Ciadamidaro et al. (2013) showed enhanced growth and survival of *P. alba* saplings following SL treatment.

The pseudototal concentrations of trace elements in the studied soil were significantly higher than the commonly accepted tolerance levels for plants, especially in the case of As (15 mg kg^{-1}), Cu (200 mg kg^{-1}), and Pb (500 mg kg^{-1}) (Mendez and Maier, 2008). The ecological and toxicological effects of contaminated soils are mainly determined by the bioavailable fraction of the contaminants. This fraction dictates the actual

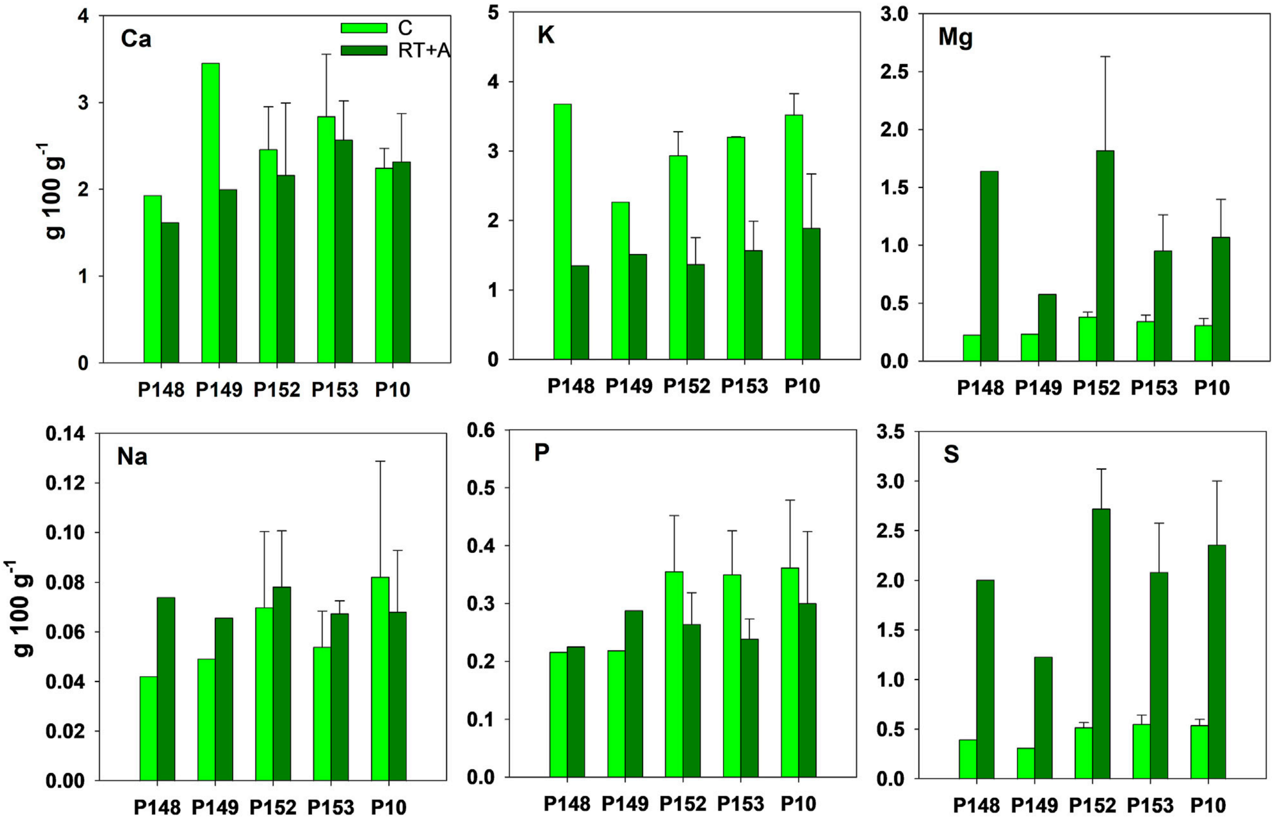

FIGURE 5 | Concentrations of macronutrients (mean and standard deviation) in poplar leaves for each hybrid control (C) and contaminated amended soil (RT + A).

exposure levels for soil organisms and the potential for these elements to move through the food chain (Chen et al., 2023). Specifically, the transfer of trace elements from soil to plants—and the related health risks—depends largely on this soluble, bioavailable fraction (Antoniadis et al., 2019).

Trace element mobility can change substantially over time as soil properties evolve. An increase in soil pH is particularly relevant, as it typically reduces the solubility of cationic metals and thereby decreases their bioavailability for plant uptake (Antoniadis et al., 2017). In the present study, SL application increased soil pH by approximately five units, substantially reducing the availability of most cationic trace elements. Notably, the bioavailable fractions of Pb and As in untreated RT soils were already low relative to their total concentrations. This reduced mobility aligns with the natural aging of anthropogenic contaminants, which typically results in decreased solubility and plant transfer potential over time (Antoniadis et al., 2017).

It is important to emphasize that As behaves differently from most trace metals. While cationic metals typically exhibit reduced bioavailability with increasing pH, As exists predominantly as an anion in soils. Consequently, As adsorption capacity tends to decrease under higher pH conditions, leading to increased mobility and potential bioavailability (Beesley et al., 2014). Consequently, pH amendments such as SL may have contrasting effects on arsenic mobility compared to other metals, influencing its environmental fate and potential uptake by plants.

Plant Tolerance and Stress Responses

Trace elements can severely impair plant growth by disrupting physiological and biochemical functions (Bharti and Sharma, 2022). This detrimental effect was evident in the poplar hybrids, where a reduced proportion of cuttings produced green leaves in the contaminated soil.

Soil acidity, nutrient deficiencies, and elevated levels of potentially toxic elements in mine soils strongly limited germination. *Brassica juncea* failed to germinate in untreated RT soil, consistent with findings from Fernández-Landero et al. (2024), who also reported poor germination of this species in acidic mine soils. Clemente et al. (2005) similarly demonstrated that soil pH strongly constrains plant growth and biomass production. In contrast, *L. purpureus* successfully germinated and survived in untreated contaminated soil, albeit with delayed emergence and reduced biomass. Its resilience is likely linked to larger seed size, which provides additional nutritional reserves during early growth stages. However, high trace element availability and low fertility, particularly potassium deficiency (threefold lower in RT than in control soils), contributed to reduced biomass, consistent with reports linking K availability to Lablab productivity (Ruthrof et al., 2018).

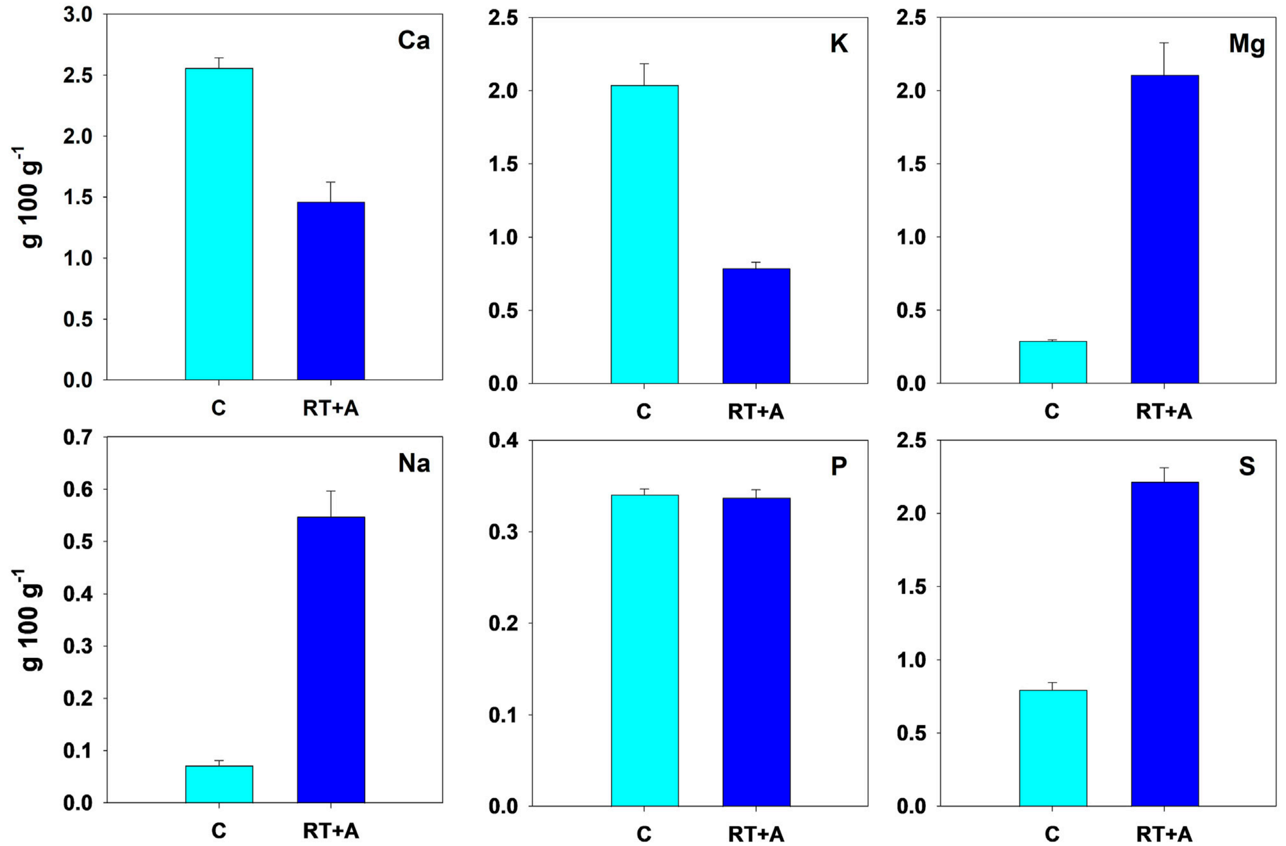

FIGURE 6 | Concentrations of macronutrients (mean and standard deviation) in aerial biomass of *B. juncea* in control (C) and contaminated amended soil (RT + A).

Trace elements are known to disrupt photosynthesis by impairing chlorophyll synthesis and electron transport (Bharti and Sharma, 2022). Surprisingly, chlorophyll content (SPAD values) did not significantly differ between poplar hybrids grown in control and RT + A soils, suggesting a potential tolerance to contaminated conditions. Similarly, *L. purpureus* showed no significant SPAD differences between RT + A and control soils, with only untreated RT soil causing reduced chlorophyll levels. This indicates that the amendment mitigated stress effects and that *L. purpureus* possesses physiological adaptations—such as efficient nutrient uptake or tolerance mechanisms—that support photosynthetic activity under contamination (Naeem et al., 2020).

Poplars, as members of the *Salicaceae* family, are well adapted to nutrient-poor soils and high trace element concentrations (Punshon, 2001). Their capacity for Cd and Zn accumulation was evident, particularly in hybrids P152 and P153, despite low soil bioavailability for these metals. Similar leaf-to-soil concentration patterns have been reported by Madejón et al. (2004), supporting the role of poplars as biomonitors. Arsenic and Pb were the dominant contaminants in the study site. Soil As concentrations exceeded those reported in similar studies (Madejón et al., 2004), whereas Pb levels were exceptionally high. Although Pb is typically immobile in soils, a meta-analysis by Tőzsér et al. (2023) showed substantial leaf accumulation in poplars, suggesting the need for further investigation into Pb uptake dynamics and remediation potential.

Brassica juncea accumulated the highest metal concentrations in aerial tissues among all studied species, especially As, Cu, Hg, and Pb in amended contaminated soil, despite the low availability caused by the addition of lime to this acidic soil (Rani et al., 2023). *L. purpureus*, while less efficient in trace element accumulation, demonstrated exceptional tolerance, growing in untreated contaminated soil without exceeding normal tissue concentrations (except for Pb in RT soils). Its leguminous nature, stress tolerance, and adaptability to challenging environments (Aguilar-Garrido et al., 2023) highlight its suitability for use as a pioneer species in phytoremediation, particularly in regions like the Iberian Pyrite Belt.

Trace metals often disrupt nutrient uptake by competing with essential macronutrients such as Ca and Mg, leading to nutrient imbalances that impair plant health (Angon et al., 2024). In this study, the low K and Ca concentrations observed in poplar hybrids under the RT + A likely resulted from the reduced fertility of contaminated soil compared to agricultural control soil, as well as competitive interactions between essential nutrients and trace metals. Nonetheless, K concentrations remained slightly below the adequate threshold (1.5%) (Reuter

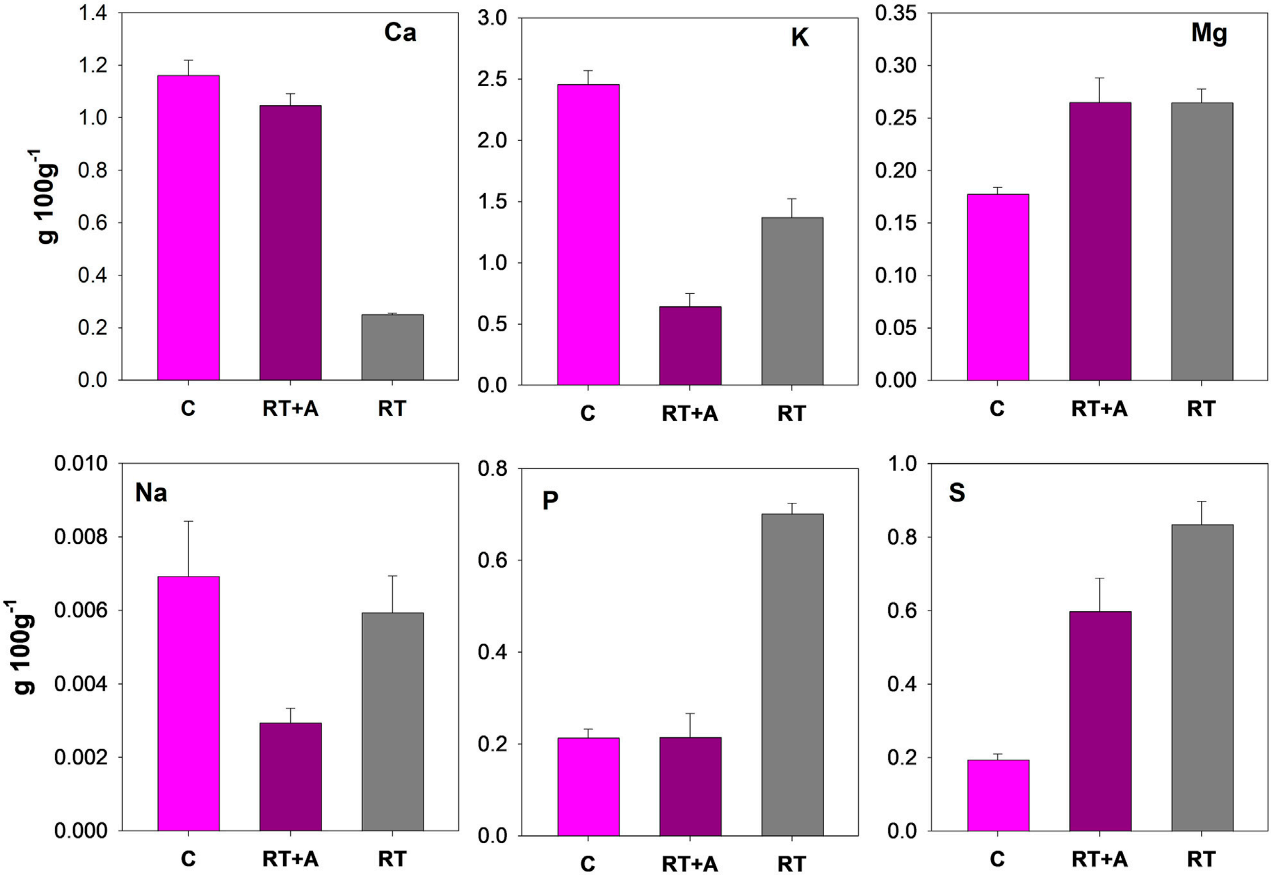

FIGURE 7 | Concentrations of macronutrients (mean and standard deviation) in aerial biomass of P. *purpureus* in control (C), contaminated amended (RT + A) and contaminated soil (RT).

and Robinson, 1997), suggesting only mild deficiency. Magnesium concentrations were elevated in contaminated soils, exceeding adequate thresholds (0.15%) and values reported in similar environments (Madejón, 2003), possibly due to enhanced uptake under stress conditions. Sulfur concentrations were also higher, reflecting naturally elevated soil S levels. Conversely, phosphorus was deficient in RT + A soils (below the 0.3% adequacy threshold), which may limit biomass production. No clear Ca uptake trend emerged, suggesting additional soil or physiological factors influence its absorption.

For *B. juncea*, nutrient concentrations were similar between soil types, although Ca and K deficiencies were evident. *L. purpureus* generally exhibited low nutrient levels, except for K, indicating potential reliance on efficient nutrient use or tolerance mechanisms for survival in poor soils. The higher concentrations of K, P, and S in RT compared to RT + A soils may stem from lower biomass that can produce a concentration effect, as well as pH-driven differences in nutrient availability within the surface soil layer.

Considerations for Phytoremediation

The optimal amendment dosage should be tailored to the specific remediation objective. When aiming to enhance phytoextraction, the amendment rate should be carefully calculated to raise soil pH only to the extent necessary to improve soil productivity, without creating overly alkaline conditions that decrease the availability of cationic trace elements. In contrast, for phytostabilization strategies, applying a higher amendment dosage to maintain a more alkaline pH is preferable, as this reduces cationic metal mobility and limits their uptake by plants. In the case of As, the increase in pH did not result in concentrations that are environmentally significant. Among the poplar hybrids evaluated, P152 and P153 exhibited the highest proportions of cuttings producing green leaves identifying them as the most promising candidates for phytoremediation applications, irrespective of the specific remediation strategy. In this study, the low availability of Cd and Zn in the soil limited their potential for phytoextraction; thus, these hybrids primarily served as bioindicators for these elements. Under conditions with greater Cd and Zn availability, however, they may be more effective for phytoextraction. For most other trace elements, with the exception of Pb, P152 and P153 appear better suited for phytostabilization approaches. Given the extremely high Pb concentrations in the contaminated site, additional research is required to elucidate Pb uptake mechanisms in poplars and to assess their potential contribution to Pb remediation.

Both companion herbaceous species, *B. juncea* and *L. purpureus*, demonstrated notable potential for phytoremediation in contaminated soils. Interestingly, *L. purpureus* exhibited strong tolerance to untreated contaminated soils, sustaining growth despite adverse physicochemical conditions. This resilience underscores its ecological value and suitability for future research. Furthermore, as a nitrogen-fixing legume, *L. purpureus* could function as a pioneer species in phytoremediation strategies, contributing to soil recovery by enhancing nitrogen availability and improving soil structure.

These results highlight the necessity of the selection of most appropriate plant species and amendments according to site-specific conditions and remediation objectives. Integrating both phytoextraction and phytostabilization approaches may provide a more efficient and sustainable solution for restoring contaminated soils.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to the study design. PM and EM interpreted the study and analyzed the data. PM, LC, MC, LS, CN-F, and EM reviewed the manuscript. CN-F, LS, PM, and EM conducted the experiments. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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