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Phytomining of Rare Earth Elements: Dynamics of Rhizosphere Processes and Element Interactions in the Soil

Nthati Lillian Monei

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology and has also been submitted to Technische Universität Bergakademie Freiberg to obtain a double doctoral degree from both Institutes.
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Haruldaste muldmetallide fütokaevandamine: risosfääri protsesside ja geokeemiliste protsesside dünaamika pinnases

NTHATI LILLIAN MONEI

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Dynamics of Rhizosphere Processes and Element Interactions in the Soil

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List of Publications

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III Wiche, O., Dittrich, C., Pourret, O., **Monei, N**., Heim, J., & Lambers, H. (2023). Relationships between carboxylate-based nutrient-acquisition strategies, phosphorus-nutritional status and rare earth element accumulation in plants. *Plant and Soil*, 1–22. doi: 10.1007/s11104-023-06049-9

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Author's Contribution to the Publications

Contribution to the Publications in this thesis are:

I The author participated in data collection, analysis and interpretation of the data, as well as writing the manuscript draft

II The author participated in data collection, analysis and interpretation of the data, as well as writing the manuscript draft

III The author contributed towards the study conception and design as well as material preparation, data collection and revising the manuscript.

IV The author contributed towards the experiments (methodology validation), data curation, visualisation and interpretation, manuscript editing and review.

Manuscripts:

I The author participated in the experimental design and performed all the experimental work. Additionally collected, analysed and interpreted the data and wrote the manuscript draft.

Abbreviations

AEC	Anion exchange capacity
ANOVA	ANOVA-Test
CEC	Cation exchange capacity
CE	Circular Economy
DW	Dry weight
Eh	Redox potential
HAS	Hydroxy-aluminosilicates
HREEs	Heavy rare earth elements
ICP-MS	Inductively coupled plasma mass spectrometry
LMOWA	Low molecular weight organic acids
LREEs	Light rare earth elements
MANOVA	Multivariate analysis of variance
pH	pH value
P_i	Inorganic P
P_o	Organic P
REE()s	Rare earth element(s)
ROS	Reactive oxygen species
SD	Standard deviation
SOM	Soil organic Matter

1 Introduction

Although rare earth elements (further denoted as REE(s)) are found in most geological settings, they are also present in previously mined sites, agricultural fields and sometimes in lean ores. Lean ores refer to low-grade ores which may either be complex or not economic to extract via conventional mining processes (Dinh et al., 2022). Furthermore, REEs are essential for many contemporary technologies, and are significant in producing high-technology electronics in fields like renewable energy, electromobility, automation technology, and military hardware (Arbalestrie et al., 2022; Ascenzi et al., 2020). On the same note, because of their widespread use in various sectors, REEs are now regarded as emerging contaminants (Dinh et al., 2022). However, this surge in demand for REEs has resulted in the exploration and operation of more REE mines, thus releasing even higher concentrations of REEs in the soil and waterbodies (Tao et al., 2022; Yan Wang et al., 2021). Additionally, phosphate fertilisers are produced from REE-rich rocks (such as monazites). Increased use of such fertilisers may increase REE concentrations in agricultural soils too (Carpenter et al., 2015). As this may be, plant and human exposure to high REE concentrations threatens well-being. As a result, taking action to remediate REE-contaminated soils and further recovering them from secondary resources have become urgent environmental challenges that have lately drawn more attention (Dinh et al., 2022; Heilmeier, 2021; W. S. Liu, Guo, et al., 2019; W. S. Liu, Laird, et al., 2021; Moschner et al., 2020).

The concept of circular economy (CE) encourages openness in shifting to more business endeavors that incorporate the exploration of the biosphere in its fullness. This ensures innovation of high-value commercial products that centrally benefit the economy and directly and indirectly promote sustainable and high-value products (Moreira et al., 2021). In this sense, the profitability of establishing a revegetation strategy on polluted old mine sites may be improved if the cultivated plants can extract commercially valuable metals from the soil (Heilmeier, 2021). Therefore, phytoremediation offers the opportunity to sustainably manage and promote recultivation on post-mining landscapes or brownfields contaminated with trace metals such as REEs (Chaney & Baklanov, 2017; Dang & Li, 2022; Gomes, 2012; Moreira et al., 2021). The exploration of phytoremediation has been ongoing for well over 65 years, using plants with high biomass yield and metal tolerance and various soil amendment methods to restore and invigorate vegetation, especially on soils with high metal contamination (Chaney & Baklanov, 2017).

The evolution of phytoremediation and the associated phytotechnologies to what it is today began as a quest to understand and explore metal-tolerant plant species and various soil amendment techniques to regenerate vegetation at metal-contaminated sites (e.g. mine wastes or smelter wastes) (Chaney & Baklanov, 2017). This being said, phytoremediation (via phytostabilization and phytoextraction) offers an opportunity, firstly to extract or stabilize labile trace elements (such as Al, Cd, Mn and REEs) thus reducing their phytotoxicity, and secondly, to further extract elements of high economic value (phytomining) through processing the harvested biomass for bioenergy, then recovering the elements from the biochar produced (Mohsin et al., 2022). Phytomining itself is a technique in which plants are used for recovering metals from lean ores, for economic gain through the cultivation, harvesting, and bio-energy processing of metal hyperaccumulator plant species. Here, the REEs are extracted from the bio-ore, which is a biproduct of bioenergy processing (Kovarikova et al., 2019).

Of course, with the development and understanding of how these phytotechnologies work, one of the main objectives for phytoremediation is cultivating hyperaccumulator species to extract economic metals such as REEs. The concept of phytomining has been extensively explored when it comes to nickel (Ni) and gold (Au) (Barbaroux et al., 2012; Chaney & Baklanov, 2017; Dang & Li, 2022; Zhang et al., 2016).

The geo-biochemical processes for the phytomining of REEs however, have yet to be fully elaborated. The main issue limiting the effectiveness of phytoextraction is thought to be the availability of elements in soil to plant roots rather than their overall concentrations. Higher plant´s roots have developed mechanisms to affect the elements' availability in soils through root-soil interactions. As a result, these interactions actively control the availability of element pools through various physical, chemical, and biological processes in the area around the root, known as the rhizosphere. Since the efficiency of phytomining depends on the availability of the target elements to the plants, it is therefore essential to investigate methods that can increase this efficiency. Most studies have focused on the use of hyperaccumulator species (C. Liu et al., 2021; W. S. Liu et al., 2020; W. S. Liu, Zheng, et al., 2021) and plants with high yield.

The work completed for this thesis focuses on how rhizosphere activities affect the bioavailability of rare earth elements to plants, and which methods can be used to enhance the availability REEs to plants. The thesis was compiled from a collective of experiments, conducted under controlled growth conditions in the laboratory and green house, as well as in the field.

The main chapters of the thesis include an overarching literature review to understand the availability of REEs in the soil, and how plants can access them, interactions between REEs and other elements in the soil, plant species capable of accumulating REEs in their biomass, factors that affect the phytoextraction of REEs in the soil, as well methods that have been used to enhance REE phytomining. The second chapter covers the materials and all the methods followed for all the experiments conducted in this thesis. Finally, the discussion of the results, which forms the general synthesis of the thesis and the main conclusions and summary of the findings.

2 Background

2.1 Availability of REEs in the Soil

Rare earth elements make up a set of 15 chemically and geochemically similar elements from the Lanthanide group, and additionally yttrium (Y) and scandium (Sc), known to occur in almost all rock formations (Kabata-Pendias, 2010; Tyler, 2004). In most cases, REEs are classified into two groups, which differ according to their atomic weight, alkalinity, and solubility. The first group is the light rare earth elements (LREE) which are made up of lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), and samarium (Sm). The second group is the heavy rare earth elements (further denoted as HREEs, HREE), made up of europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu) (Dinh et al., 2022; Tyler, 2004).

However, despite what their names would imply, these elements are not as “rare”, concerning their occurrence in the Earth’s crust. The term refers to the complexity of the metallurgical processes involved for successfully extracting and separating individual REEs species (Dinh et al., 2022; W. Li et al., 2022). However, depending on the location and geology, the concentration of REEs in the soil might vary significantly, indicating their availability at considerable quantities in the Earth’s crust (Wiche, Zertani, et al., 2017). In terms of quantifying the amounts and concentrations of REEs available in the earth’s crust, La, Ce, Nd, and Tm are known to be found on average, at 35 $\mu g\ g^{-1}$, 66 $\mu g\ g^{-1}$, 28 $\mu g\ g^{-1}$ and 0.5 $\mu g\ g^{-1}$, respectively (Ramady, 2008; Ramos et al., 2016; Tyler, 2004). This being said, Ce is the most abundant REE and is available in quantities similar to those of known micronutrients copper (Cu) and zinc (Zn) (Haynes,2016; Dinh et al. 2022). Furthermore, REEs are more concentrated in areas with weathering or erosion, as the elements can be leached out of the parent rock and accumulate in the soil (Li, 2018).

In the natural distribution of the REEs, the Oddo-Harkins rule can be seen, where even-numbered REEs (Ce, Nd, Sm, Gd, Dy, Er and Yb) are more abundant than odd-numbered REEs (La, Pr, Eu, Tb, Ho, Tm, and Lu) (Tyler, 2004). The affinity of REEs to oxygen makes them more prone to be associated with minerals such as carbonates, fluorides, phosphates, and silicates. Among the most known minerals, REEs are mostly associated with: bastnaesite ($(Y, Ce, La)CO_3F$), monazite ($(Ce, La, Y, Th)PO_4$), or xenotime (Y-Nitrate) (Ramos et al., 2016; Tyler, 2004). Furthermore, LREEs are typically associated with minerals like apatite rich in Ca^{2+} and PO_4^{2-}. The highest concentrations of REEs among phosphatic minerals are found in monazite, with the LREEs being preferentially absorbed into its structure, while the HREEs are preferentially integrated in xenotime's structure. (Ramos et al., 2016).

2.2 The Mobility of REEs in the Soil

To comprehend the environmental accessibility of REEs for potential anthropogenic pollution in the future, multiple studies document their mobility in unpolluted natural soil-plant systems. Most of the studies of REE bio-geochemistry, include their content and concentrations in plants, chemical forms in which they are available to plants, distribution, transportation, and accumulation in the soil-plant system (Dinh et al., 2022; Heilmeier, 2021; Kovarikova et al., 2019; Wiche et al., 2015).

The availability of REEs in the soil, just as with all elements, is not only determined by the parent rock, but also the bio-geochemical processes governing the weathering of

minerals greatly influence their availability in the soil (Brioschi et al., 2013; Kabata-Pendias, 2010). This means, soil REE concentrations do not reflect the exact concentrations in the parent rock. Soil properties such as pH and cation exchange capacity (CEC) typically affect the adsorption of La, Y, Pr, and Gd to soil particles. Additionally, decreased pH and redox potential increase the availability of La, Ce, Gd, and Y (further discussed in Section 2.4) (Jingjing Liu et al., 2014; Ou et al., 2021). This being said, the mobility of REEs is higher at pH 3.5 to 5.5. Beyond that, REE mobility decreases. This is because REEs form complexes with carbonates, fluorides, phosphates and sulfates which restrict REE bioavailability (Kabata-Pendias, 2010; Khan et al., 2017; Tyler, 2004). The formation of complexes with carbonates leads to increased REE mobility, but is reduced when REEs are complexed with phosphates (Galhardi et al., 2022; Ramos et al., 2016; Tyler, 2004). Consequentially, REEs are demonstrated to be more water soluble as complexes with ions such as bromate (BrO_3^-), chloride (Cl^-), perchlorate (ClO_4^-), nitrates (NO_3^-), but REE complexes with phosphates (HPO_4^{2-}, and PO_4^{3-}) are said to be highly insoluble (Lima & Ottosen, 2021). In these complexes, REEs are more insoluble in the soil, especially at neutral pH conditions. However, as the pH decreases (up to pH 5) REEs are bound to Fe-oxyhydroxides, and a further decrease in pH would result in the dissolution of REEs (Chen et al., 2022). Iron and sulfate in particular, are more prone to form secondary minerals which are closely precipitated with REEs, as well as other metals such as Al and Cd and in such conditions increased sulfate concentrations would lead to accelerated REE sorption (Chen et al., 2022). Additionally, in terms of binding in the soil, REEs form complexes with Fe, Al, and Mn, showing that at decreased pH conditions the Mn-, Fe-, and Al- oxides are the main available compounds to bind with REEs (Lima & Ottosen, 2021; Tyler, 2004).

Heavy REEs are said to have a high affinity towards chelates and ligands, compared to LREEs, which normally causes the high LREE/HREE ratio in the soil solution (Marsac et al., 2021; Miao et al., 2008). Factually, at circumneutral to partially alkaline pH the concentrations of Al^{3+} and Fe^{3+}, (competing with REEs for binding sites on the soil organic matter, SOM), decrease. This allows REEs to form complexes with chelates and ligands. Because they are more mobile than HREEs, which form highly stable complexes in the soil, LREEs are reported to be enriched in the shoots of some plant species, increasing their chances of being extracted from the soil (Brioschi et al., 2013). The formation of complexes with carbonates in alkaline conditions leads to increased REE solubility, where these REE-carbonate complexes ($REECO_3^+$ and $REE[CO_3]_2^-$) are significantly higher in concentrations than organic substances in the soil solution – thus outcompeting the organic matter for REE binding. Contrarily, in acidic conditions, the competitive cations Al^{3+} and Fe^{3+} are more abundant, increasing their capacity to bind to the chelates and ligands, leaving carboxylic as the only site free for binding with REE. Similarly in these acidic conditions, dissolved REEs and REE-sulfate complexes ($REE\text{-}SO_4^+$) are the most dominant, which are of reduced REE-mobility; this also corresponds to REE-phosphate complexation (Galhardi et al., 2022; Marsac et al., 2021; J. Tang & Johannesson, 2003).

2.3 The Uptake, Distribution and Contents of REEs in Plants

Effective phytoextraction and accumulation of REEs depend highly on the bioavailability of metals. This means that the concentration and speciation of REEs in the soil, the most significant determinants of plant absorption of these elements than the plant itself. Although REEs can be available in high concentrations in some soils, the main challenge is the available fraction of these elements that can be extracted and accumulated in plant biomass. For instance, Wiche et al. (2017) demonstrated that only up to 30% of the REEs in

soil could be accessed by plants. From sequential extraction, it has been shown that only the water-soluble, exchangeable and carbonate-bound REE fractions are easily accessible to plants (F. Li et al., 1998; Wiche, Zertani, et al., 2017). The characterisation of REEs has been linked to the potential to select organic and inorganic ligands which enable both REE complexation and effective phytoextraction of REEs from the soil (Grosjean et al., 2019).

Rare earth elements are taken up in plants through the exact mechanisms responsible for nutrient influx in plants. In this sense, the bioavailability, absorption, transportation, and accumulation of REEs in plants cannot necessarily be separated from other elements, especially micro- and macronutrients (Kovarikova et al., 2019). Although REEs are not recognised as essential nutritional elements for plants, their uptake occurs through Ca, Na, K and Al channels (W. S. Liu, Zheng, et al., 2019; Rengel, 1994). Heavy REEs and Al^{3+} are taken up through the same pathways, whereas LREEs are absorbed by the roots via Ca-ion channels (Chen et al., 2022; Kabata-Pendias, 2010; Yuan et al., 2017). This suggests that plants generally take up REEs and nutrients almost indiscriminately through the roots; translocating and depositing them in the leaves (Gu et al., 2002).

The uptake and transportation of REEs occur passively through the apoplastic pathways, where they are transported by mass flow against the diffusion gradient. In other cases, REEs can be taken up actively through symplastic pathways as solutes, where they are carried through the plasmalemma. where the ions enter through the cytoplasm (Brioschi et al., 2013). When uptake occurs, the REEs initially have to cross through the Casparian strip, which can constrict REE translocation into the central cylinder and the transpiration stream, thus reducing the amounts of REEs deposited in plant shoots (Brioschi et al., 2013; Ozaki & Enomoto, 2011; Ramos et al., 2016). According to Li et al. (1998) and Brioschi et al. (2013), the sequence of REE contents in various plant sections was root > leaf > stem > grain, as demonstrated in *Hordeum vulgare* (barley).

The absorption of REEs can also occur through direct application on the leaves. In such cases, the apoplastic barriers still act as constricting factors, at such limiting REE distribution to other plant tissues, which would lead to REEs being highly concentrated in leaves, followed by stems > roots > fruits (Brioschi et al., 2013; Ramos et al., 2016). The rhizosphere plays a significant role for REE uptake in plants as most of the crucial element-plant root-microbe interactions occur therein. This leads to physico-chemical alterations of the elements available in the soil increasing their bioavailability for plant uptake (Semhi et al., 2009). Rare earth element concentrations are often lower in the plant biomass compared to soil concentrations, indicating that these concentrations are not dependent on the soluble forms. This can be evaluated through the translocation factor, which is the ratio of the elements in plants to the concentration in the soil. The concentrations of REEs in plants usually depend on their radii and atomic numbers. Generally, REEs can be found in plant tissues at modest concentrations in quasi-contaminated environments even though they are not essential to plants (F. Ding et al., 2022). The concentrations of REE in plants can range from less than 1 g/kg to more than 15 mg/kg (D.W) (S. Ding, Liang, Zhang, Wang, & Sun, 2006; Gu et al., 2002; Kabata-Pendias, 2010; Tyler, 2004). In plant leaves, REE concentrations have been found at 0.0011 (Lu), or 0.33 mg/kg DW (Ce) and even as high as 5 mg REE/kg DW (S. Ding, Liang, Zhang, Wang, & Sun, 2006; Fehlauer et al., 2022; Wiche, Székely, et al., 2016). Furthermore, LREEs show a higher enrichment in plants as compared to HREEs (Kabata-Pendias, 2010; Semhi et al., 2009). This separation of LREEs relative to other REEs may reflect the function of ligands in the complexation of LREEs, causing an increased LREE mobility compared to HREEs, which can be complexed in the soil (Semhi et al., 2009).

2.4 Interaction of Rare Earth Elements with other Elements in the Rhizosphere

In most cases, REEs can have synergistic or antagonistic relationships with other elements, especially nutrients. Although REEs are not recognised as essential nutritional elements for plants, their uptake occurs through Ca, Na, K and Al channels (W. S. Liu, Zheng, et al., 2019; Rengel, 1994). Several of the REEs can compete with Ca (especially La and Ce). Rare earth elements have trivalent charges, hence a higher charge density, which compete with and replace Ca at Ca-binding sites in biological molecules, resulting in an antagonistic relationship between them (Thomas et al., 2014; Tyler, 2004). In this sense, La is capable of replacing Ca on the cell wall binding sides, substituting the biochemical function of Ca and other Ca-mediated processes, while inhibiting Ca translocation and efflux in plants (Diatloff et al., 2008). Therefore, these interactions can account for part of REE phytotoxicity (Kovarikova et al., 2019; Thomas et al., 2014).

Similar to REEs, Al also inhibits Ca^{2+}, which was observed on the *Amaranthus tricolor*, as it also competes for binding sites with Ca^{2+} on the plasma membrane. Rengel (1994) investigated the interaction between Al and REEs (La, Gd, Ce, In, and Sc) and how they affect Ca^{2+} uptake in *A. tricolor*. The findings demonstrated that firstly, the pH plays a key role in these interactions. At low pH (pH 4.5), Al was antagonist towards Gd, as it also increased Ca inhibition. However, Al also behaved in an antagonist way towards La, and alleviated Ca inhibition that occurred due to the La treatment (Rengel, 1994). This suggests that Al and La ions get bound to the same binding site.

Liu and colleagues (2021) investigated the co-accumulation of REEs and Al and Si in *Dicranopteris dichotoma*. The results from this study indicated the involvement of Si, in the uptake of Al and REEs in plants. Furthermore, there was a positive correlation between the accumulated Si and Al, as well as Al and REEs, suggesting similar accumulation behaviour for these elements (W. S. Liu, Laird, et al., 2021). Similar results have been reported for *Phytolacca americana*, which accumulated high concentrations of Al, REEs and Mn, showing a positive correlation between REEs and Al and Fe (C. Liu et al., 2021). Furthermore, because REEs and Al have identical charges (3+), the use of REEs to investigate the phytotoxicity of Al has become common (W. S. Liu, Zheng, et al., 2021; Yuan et al., 2017).

Some plant species that are well able to respond to nutrient deficiency, especially P- and Fe-deficiency. For instance, leguminous forbs such as *Lupinus albus* and *Lupinus angustifolius* release increased amounts of carboxylates or develop cluster roots in response to P-deficiency (Lambers, 2022; Lambers et al., 2013; Ligaba et al., 2004). The released carboxylates release both organic and inorganic P (P_o and P_i), which are further sorbed onto soil particles. In this process, the carboxylates replace phosphate in the soil, rendering it more soluble and mobile, which is further hydrolysed by phosphatase and taken up to the roots via P-transporters (Lambers et al., 2013). While P is mobilised, other micronutrients (especially Fe, then Cu and Mn) are also chelated as they are mostly bound to P-compounds. The chelated Fe^{3+} migrates to the root surface, becomes reduced (Fe^{3+} to Fe^{2+}) in the plasma membrane, and further transported to other plant tissues via Fe-transporters (Honvault et al., 2021; Lambers et al., 2013). As this may be, the release of carboxylates can therefore not be limited to the increased mobility of P and Fe, but to the alteration of the solubility of other non-essential elements

such as REEs, Cd and Pb. *Cicer arietinum* (chickpea) is also known to release carboxylates as a response to P-deficiency, which can result in the mobilisation of REEs via complexation with ligands (Pang et al., 2018; Wiche, Székely, et al., 2016).

2.5 Effects of REEs on Plants

Although not regarded as essential for plants, REEs can be beneficial for the growth and development of plants, and this varies depending on the plant species (Tao et al., 2022). At low concentrations (<0.5 mg REE kg^{-1} soil) REEs can promote seed germination and enhance seedling development, but at high concentrations the effects become detrimental (Thomas et al., 2014). For instance, the administration of La and Ce at 0.5 mg kg^{-1} was reported to promote root developments of *Arabidopsis thaliana* but did not further increase the overall plant yield as the plants grew further (He & Loh, 2000). According to Diatloff et al. (2008), plant treatment with La and Ce to corn (*Zea mays*) or mungbean (*Vigna radiata*) cultivated in a hydroponic system did not improve plant growth and even showed growth restriction at Ce doses higher than 5 µmol L^{-1}. Moreover, it has been shown that REEs boost the growth of crops like wheat and rice (Hu et al., 2004). However, the beneficial effects of REEs on plant development may be limited to certain growth phases or soil conditions (Tao et al., 2022). Other benefits of REEs in plants include speeding up photosynthetic activity, improving enzyme activity, and increasing nutrient uptake. These results imply that REEs have potential benefits for plants and agriculture.

2.6 Factors Affecting the Uptake and Accumulation of REEs in Plants

The concept of phytoextraction, a subprocess of phytoremediation, through which plants extract and translocate elements from the soil to the (above-ground) plant biomass, has been explored since its introduction by Chaney and Hornick (1977). The investigation of REE uptake has been conducted on multiple plant species (Brioschi et al., 2013; Carpenter et al., 2015; S. Ding, Liang, Zhang, Huang, et al., 2006; C. Liu et al., 2021; W. S. Liu, Laird, et al., 2021; Wiche & Heilmeier, 2016). To achieve a successful phytoextraction or accumulation of rare elements, it is crucial to take note of the constant transformation of soil conditions, and plant morphological changes which also influence how REEs are available to plants. Figure 1 (p.19) highlights the factors that can affect the bioavailability and accumulation of REEs in soil-plant systems.

Soil physical and chemical properties play a major role in the uptake of elements (Wiche et al., 2015). Soil pH significantly affects element mobility and availability from the soil to plants. Cao et al, (2002) demonstrated that the higher the soil pH was, the lower were REE concentrations in *Triticum aestivum* L. (wheat). Similarly, Gu et al (2002) also confirmed that at pH 6.7, LREEs were adsorbed, contrariwise at pH 4, only HREEs were absorbed in wheat roots. This is because at low pH REE leaching occurs in the rhizosphere, releasing the insoluble fraction of REEs, making them available to plants (Cao et al., 2002). Alongside pH, the redox potential also influences REE bioavailability, in that, with decreasing redox potential, the mobility of REEs is increased (Cao et al., 2001). Cation exchange capacity (CEC) influences the adsorption and desorption of elements from the soil solution to the plant biomass (Kovarikova et al., 2019). Cation exchange capacity acts as a direct indicator of the soil's cation buffer capacity. High CEC results in enhanced cation retention, which reduces the absorption of those metals by plants (Cheng et al., 2015; Xiao-ping Wang et al., 2004).

Another factor related to the soil properties are humic acids, the bioavailability of REEs increases when humic acid is relatively low in the soil, whereas increased humic acid concentrations will lead to decreased REE bioavailability (Gu et al., 2002; Kovarikova et al., 2019; Xueyuan et al., 2001). This is because top-soils are usually rich in organic matter derived from decomposed plant material which eventually gets bound to clay minerals' surfaces (Ou et al., 2021).

Soil organic matter (SOM) also plays a role in the bioavailability of REE. According to Miao et al. (2008), dissoluble SOM can interact with REEs to generate dissoluble organic ions, which are subsequently transported by subterranean drainage, altering how REEs behave chemically in the topsoil. The organic matter forms strong chemical bonds with cations (including the trivalent REE^{3+}, Al^{3+} and Fe^{3+} from clay minerals) resulting in diverse organic-inorganic colloidal compounds (Marsac et al., 2021). Therefore, with increased concentrations of organic matter in the soil, more of these organic-inorganic compounds are formed further polymerising via adhesion, forming aggregates with multiple negatively charged groups. These agglomerates have a high propensity to form complexes capable of adsorbing or chelating REEs (depending on the pH), thus having a significant role in the distribution of REEs (Ou et al., 2021).

Organic soil amendments also regulate the microbial activity in the soil, as they can act as sources of energy and carbon for microorganisms. The microorganisms would then contribute towards the biogeochemical cycling of REEs, either by releasing them in the soil or immobilising them sorption or precipitation (Davranche et al., 2015). Schwabe et al. (2021) indicated that plant growth promoting rhizobacteria (PGPR) such as *Arthrobacter oxydans* and *Kocuria rosea* are able to secrete secondary metabolites which mobilise and increase the bioavailability of REEs. Shan et al. (2003) reported that REE uptake, especially LREEs, can be highly influenced by the presence of histidine and organic acids. In *D. linearis*, an increase of up to 78% for LREE concentrations was observed in the presence of histidine and organic acids. In this case, the key role of histidine was predicted to be the stimulation of LREE sequestration in the soil forming histidine-REE complexes ($RE(His)(NO_3)_3 \cdot H_2O$). Alternatively, organic acids mainly enhance the mobilisation of LREEs, making the elements readily available for uptake in plant roots (Han et al., 2005; Shan et al., 2003).

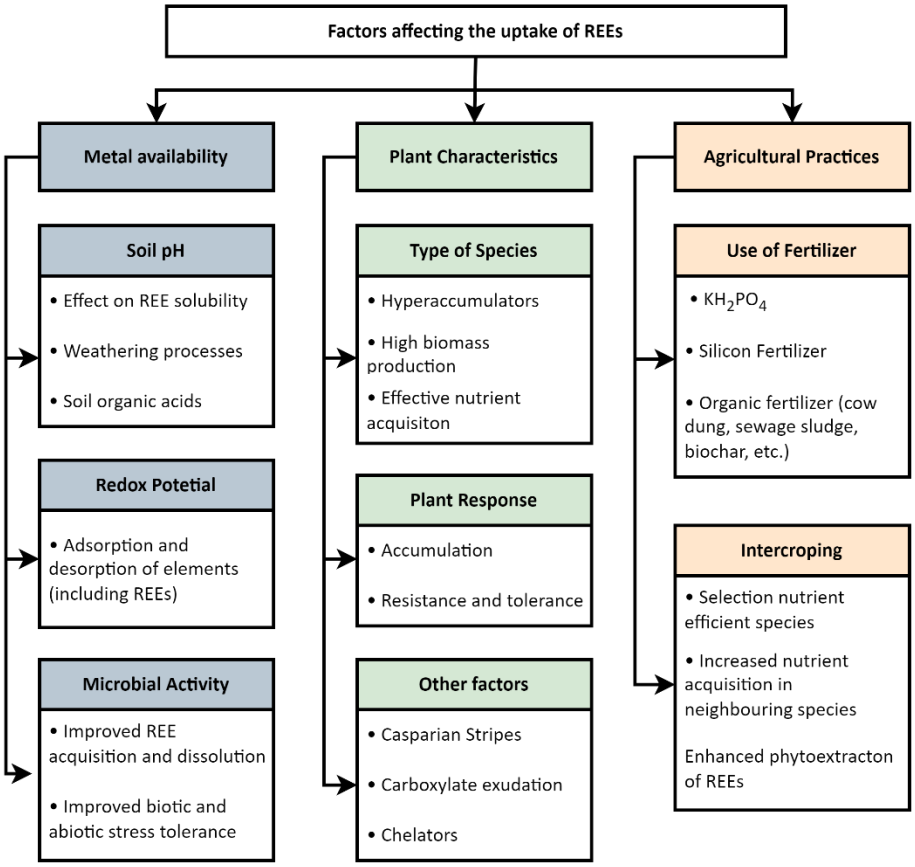

Figure 1 *Factors affecting the uptake of REEs.*

One of the most fundamental steps in establishing methods for the phytoextraction of REEs includes the identification of suitable species, based not only on their hyperaccumulation capabilities but also their ability to mobilize and accumulate elements that are often associated with REE availability. Plant species that have the ability to specifically accumulate high concentrations of specific elements, are called hyperaccumulators. For a plant to be considered as a REE hyperaccumulator, a threshold concentration of up to 1000 μg g^{-1} has been established (Wei et al., 2001).

Among the pool of hundreds of plant species that have been identified as metal hyperaccumulators, 20 of these species are said to be REE accumulators. These species include *Phytolacca americana* and *P. icosandra*, *D. linearis*, *Blechnum niponicum*, *Woodwardia japonica*), *Carya cathayensis*, *C. glabra*, *C. tomentosa*, *Pronephrium simplex*, *P. triphyllum* (Dang & Li, 2022; Khan et al., 2017; W. S. Liu, Zheng, et al., 2019). In a study by Chao and Chuang (2011), both *D. linearis* and *D. dichotoma* were found to not only hyperaccumulate REEs at highly contaminated soils but also on soils with average REE concentrations, accumulating up to 660 μg g^{-1} in leaves. Another species of interest that has been discovered is *Pronephrium simplex*, capable to accumulate 1.2 mg g^{-1} REE per dry weight (Lai et al., 2005). Additionally, Wiche and Heilmeier, (2016), also highlighted that forbs such as *Brassica napus*, *Lupinus albus, Lupinus angustifolius,*

and grasses such as *Zea mays* can be potential REE accumulators, especially due to their ability to mobilize Fe and P in the soil-root interface. Especially *Z. mays* has been used to investigate the phytoaccumulation of REEs (Heilmeier & Wiche, 2020). Xiao et al. (2003) also reported that *Blechnum orientale* showed to accumulate REE concentrations of 1022 µg g^{-1} in the plant leaves. Similarly, *Cyperus rotundus* also was shown to have significant capabilities to transport REEs, particularly LREEs, from the soil to the root and roots to the other plant organs, when cultivated on an shut down mining area (Khan et al., 2017).

2.7 Approaches to Enhance the Bioavailability and Phytoextraction of REEs

It should be noted that, in some instances, REEs are available in trace concentrations and therefore ways to accelerate the bioavailability and mobilisation of REEs even in minute quantities are required. The most common ways include the application of synthetic aminopolycarboxylic acids such as ethylenediaminetetraacetic acid (EDTA), glutamic acid, ethylenediamine disuccinate (EDDS), histidine, citric acid, and malic acid (Marsac et al., 2021; Meers et al., 2005; H. Tang et al., 2017, 2017). When applied, EDTA and EDDS increase the acid extraction fraction of heavy metals (Heilmeier, 2021). The complexation of REEs with these ligands renders them more accessible to plant roots, thus increasing their concentrations in plant tissues (Ali et al., 2013; Gmur & Siebielec, 2022). Additionally bioinoculants (such as bacteria and mycorrhiza) contribute significantly towards element absorption in plants (Gmur & Siebielec, 2022; Heilmeier, 2021; J.-W. Wu et al., 2013). Wu et al. (2009) reported a positive correlation between high glutamic concentrations and the concentrations of La and Y accumulated in *Phytolacca americana*. According to Shan et al. (2003), histidine and organic acids promote the desorption of LREEs from the soil, increasing their availability in the soil solution thus having high concentrations transferred to the plant biomass. From these results it was reported that the addition of histidine led to increased LREE concentration by up to 34% compared to reference plants (Shan et al., 2003). Citric acid together with desferrioxamine B (DFO-B) enhanced the desorption of REEs in the soil solution thus increasing their uptake in *Phalaris arundinacea*. This was related to the formation of REE-citric acid soluble complexes, which increase REE mobility, enhancing their potential in the phytoextraction of REEs (Wiche, Tischler, et al., 2017), Table 1, p.24.

Naturally, plants adapt to the constantly changing soil conditions by interacting with already present microorganisms in the soil, resulting in a soil environment that is conducive for effective nutrient acquisition and/or accumulation of other elements. Arbuscular mycorrhizal (AM) fungi are capable of enhancing plant tolerance towards biotic and abiotic stress. The availability of AM in the soil also increases not only nutrient uptake but REE transport too (Kovarikova et al., 2019). Similarly, plant growth promoting bacteria (PGPR) improve element bioavailability and mobility through several processes, including the release of chelating agents which induce rhizosphere acidification (Jalali & Lebeau, 2021; Okoroafor, Mann, et al., 2022). In a field experiment, Okoroafor et al. (2022) investigated the effects of bioaugmentation with *Bacillus amyloliquefaciens* FZB42 on the accumulation of REEs and other trace elements in *Z. mays* and *Helianthus annus*, and the result indicate increased accumulation of REEs, especially in *H. annus,* Table 1, p.24.

Besides these interactions with microorganisms, plants also acidify the rhizosphere through the secretion of natural chelates, low molecular weight organic acids such as phytosiderophores and carboxylates (e.g., citric, fumaric, and maleic acid) (Lambers, 2022). These carboxylates can mobilize nutrients as well as REEs in the rhizosphere (Gmur & Siebielec, 2022). For instance, citric acid and malic acid are said to stimulate the accumulation of La in barley (Han et al., 2005). Carboxylate secretion results in increased hydrogen ions in the rhizosphere, which reduce the pH of the soil. At reduced pH levels, the metal ions bound to soil particles are replaced causing them to be readily available for plant uptake (Lambers et al., 2013). Leguminous plants such as *Lupinus species* are capable to release citric and malic acid, which increase the availability of sparingly available nutrients (especially P) through the release of carboxylates (Lambers et al., 2013; Lambers et al., 2015). Phosphorus acquisition has been closely related to the uptake of REEs in plants (Lambers, 2022; Wiche, Székely, et al., 2016).

This being said, the low availability of REEs in the rhizosphere also opens an avenue to explore agronomic practices applied to improve the phytoextraction of not only REEs but even other trace elements. It is not only application of artificial soil amendments that can influence the uptake of REEs in plants, but agronomic methods such intercropping and crop- rotation can increase the mobility of trace elements, including REEs in plants (Heilmeier & Wiche, 2020).

This suggests selecting species with unique nutrition strategies for elements such as P-acquisition, Fe-strategies, and Si-accumulation as these may be competitive in terms of acquiring and accessing REEs in the soil along with these nutrients (Heilmeier, 2021). *Lupinus albus* (white lupin) was found to be one of the species able to accelerate phytoextraction of trace metals in either crop rotation or intercropping systems. Fumagalli et al. (2014) assessed seasonal crop rotation of hemp (*Cannabis sativa* L., a heavy metal accumulator) with white lupin (as a potential green manure) on increasing the phytoextraction capacity of copper (Cu), lead (Pb), nickel (Ni), chromium (Cr) and zinc (Zn) on alkaline soils. The findings proved that this crop rotation system may be beneficial in term of enhancing phytoextraction, as the lupin increased the availability of the trace metals (Egle et al., 2003; Fumagalli et al., 2014). Furthermore, in a field study, Wiche et al. (2016), demonstrated that intercropping *Avena sativa* L. (oats) with P-efficient species *L. albus* can increase REE uptake. This is mainly because white lupin is able to release organic acids such citrate and malate, as well as other protons in the root-soil profile thus boosting phosphorus and trace element (such as REEs and Cd) mobility and availability (Lambers, 2022; Wiche, Székely, et al., 2016).

Okoroafor et al, (2022) investigated the effect of liming and of organic fertiliser (cow and horse dung) and the results thereof indicated organic fertiliser leads to increased REE concentrations in *L. albus* and *Z. mays*. This was attributed to the hypothesis that REE recycling is more rapid in organic soils (Okoroafor, Kunisch, et al., 2022). Liming on the other side led to the reduction of the accumulation of REEs in plants, indicating that liming increases Ca in the soil, further increasing the soil pH and the chances of the formation of REE precipitates, which in turn reduces the mobility and availability of REEs to plants (Okoroafor, Kunisch, et al., 2022).

While not yet shown to be a plant-essential element, silicon (Si) is widely recognised as a beneficial element for plant growth and development. Si may reduce both biotic and abiotic stress, with abiotic stresses including stressors such as drought, heat, cold, metal toxicity, and nutritional imbalance (Greger et al., 2018; Pavlovic et al., 2021). The use of Si for the alleviation of metal toxicity, and increasing plant tolerance, has been explored

and reviewed, including its interaction with other elements such as P, Ca, Cd, and Al (Adrees et al., 2015; Brackhage et al., 2013; Jian Liu et al., 2013). However, only a few studies highlight the interaction between Si and REE (W. S. Liu, Zheng, et al., 2019). Wang et al. (2004) demonstrated that Si increased Al -tolerance in *Zea mays*, inferring that this may be a result of Si and Al forming hydroaluminosilicates (HAS) (Hodson & Evans, 2020; Yunxia Wang et al., 2004). Since REEs and Al are trivalent, it can be hypothesised that they behave in a chemically similar way. Therefore, when exposed to Si, REEs will also form complexes with Si, further resulting in increased concentrations of REEs in plants (W. S. Liu, Zheng, et al., 2019).

Since the main issue affecting the phytoextraction of target elements (in this case REEs) is their bioavailability, it is worth exploring other processes that can improve REE bioavailability towards plants. Furthermore, it would be beneficial to assess the role of other factors including plant nutrition strategies, interactions between elements in the rhizosphere, substrate properties and the application of fertilisers on the the solubility and bioavailability of REEs.

Table 1 *Previous studies that investigated the effects of different soil amendment and treatments on the accumulation of REEs in plants.*

Plant Species	Substrate	Amendment/	Outcome	References
Phalaris arundinacea	Road construction site, silty loam pH 7.8, Germany	Compost	↑REEs with compost	Moschner et al. (2020)
	Post mining site pH 6.6, Germany		↑ REEs	
Phalaris arundinacea	Clayey-silt with high REEs, Germany	Citric acid and desferrioxamine	↑REE sorption ↑REE dissolution	Wiche et al. (2017)
Triticum aestivum	Black Soil, China	Fluvic acid	↑ REE bioavailability	Zhimang et al. (2001)
Triticum aestivum	Red soil, China	Humic Acid	↑ REE in roots than shoots	Xueyuan et al. (2001)
Triticum aestivum	Black Soil and Yellow soil, China	EDTA	↑REE accumulation in shoots	Lihong et al. (1999)
Triticum aestivum	Clayey-silt, Germany	Intercropping with *L. albus*	↑ REE	Wiche et al. (2016)
Zea mays and Helianthus annuus	Agricultural Fields, Germany	*B. amyloliquefaciens* FZB42 (Rhizovital)	↓ΣREEs (17%) ↑ΣREEs (15%)	Okoroafor et al. (2022)
Zea mays *Lupinus albus* *Brassica napus*	Soil from TU Bergakademie Campus, Freiberg, Germany	Cow dung + horse dung as organic fertiliser	↑REEs in *L. albus* (53%) and *Z. mays* (46%) ↓ ΣREEs 35% in B. napus	Okoroafor et al. (2022)
Dicranopteris dichotoma	REE ore deposit (China)	Liming Histidine, malic acid, and citric acid	↓ΣREEs in all species ↑REEs (La, Ce, Pr, Nd)	Shan et al. (2003)

Aims of this Thesis

The present thesis is a synthesis of detailed research in the field of bioremediation, focusing on the phytomining of rare earth elements. Since most publications have reported ways in which soil amendments and chelators may affect the efficiency of the uptake of REEs in plants, most of them focused on plant species that have already been identified as REE hyperaccumulators. However, the most significant factor for successful phytomining of REEs is bioavailability. Therefore, it is crucial to understand processes in the rhizosphere that influence the availability of REEs to plants. The aim of this thesis is to investigate rhizosphere processes and element interactions affecting the bioavailability of rare earth elements in phytomining. Furthermore, to explore the effects of plant nutrition acquisition strategies affecting the availability and accumulation of rare earth elements in plants, the application of P-fertiliser and Si fertiliser, as well as assistive revegetation strategies such as intercropping. The data analysed in the specific studies was acquired through laboratory controlled and field studies to identify and further understand how these nutrition acquisition strategies and the absorption efficiency of essential elements may affect the bioavailability of REEs in plants.

Research Questions

1. What influence do substrate properties have on the uptake and availability of REEs?
2. What are the main interactions between phosphorus acquisition strategies and the uptake of REEs in plants?
3. What is the role of the release of carboxylates on the uptake of REEs?
4. What influence does soil amendment with Si have on the uptake of REEs in plants with different nutritional strategies?
5. How are REEs distributed in plants tissues?

3 Methods and Materials

To explore strategies for phytomining and the rhizosphere interactions that support successful uptake of rare earth elements and nutrients and trace elements in plants, this thesis is based on three different case-studies (*Publication I*, *Publication III*, *Publication IV,* and *Manuscript I*). Each of the experiments followed is thoroughly explained in the respective Publications.

3.1 Plant Cultivation

The first experiment (*Publication I*) is based on a field experiment carried out at the Bauer Umwelt Business Hirschfield (Saxony, Germany). *Hordeum vulgare* L. cv. Modena (barley) was cultivated in an intercropping system with *Lupinus. albus* cv. Feodora (white lupin) and *L. angustifolius* cv. Sonate (narrow leaf lupin) in a replacement model as illustrated in Figure 2 and further described in *Publication I*. The plants were cultivated on two different substrates, Substrate A (pH = 7.8, in yellow on the diagram), and Substrate B (pH = 6.6), both classified as silty loam. The plants were treated with the Hoagland solution with either 200 µmol L^{-1} P (NPK) or 20 µmol L^{-1} P (NK).

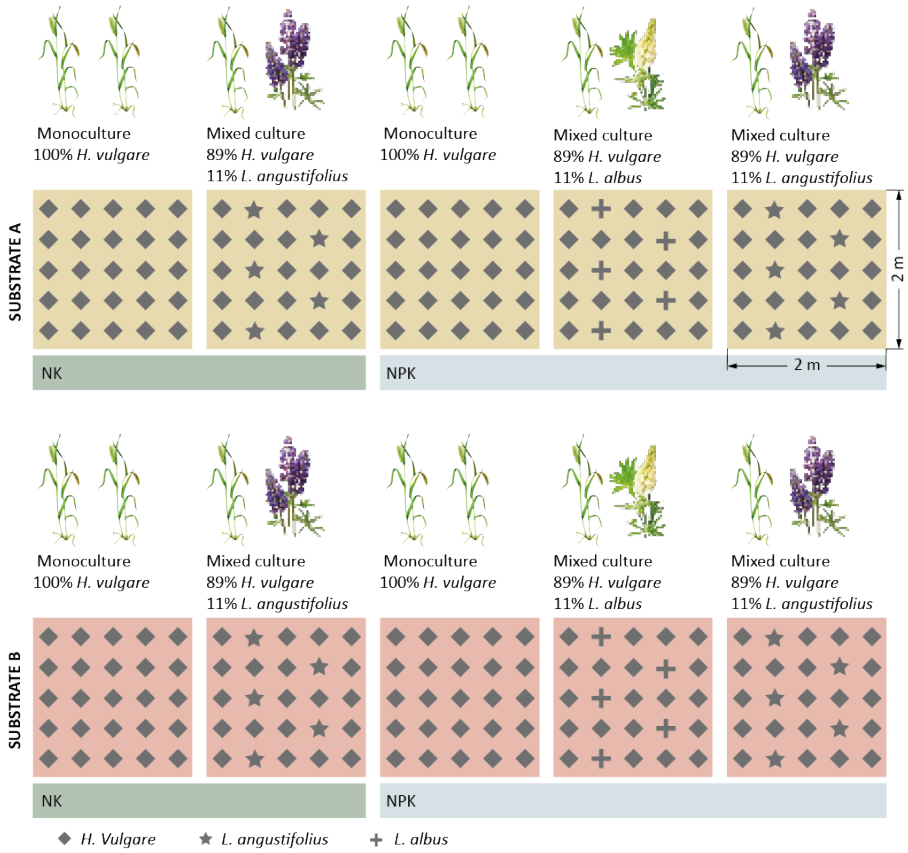

Figure 2 *Experimental setup for intercropping Hordeum vulgare with Lupinus albus and Lupinus angustifolius on two different substrates, Substrate A (yellow) and Substrate B (orange), treated with NK and NPK.*

In addition to the field experiment, a separate greenhouse experiment was conducted to further characterize the root exudates released from *L. albus* and *L. angustifolius*. The plants were treated with a nutrient solution with 20 µmol L^{-1} K_2HPO_4 (Low P, reference) and 200 µmol L^{-1} K_2HPO_4 (high P) explained in detail in *Publication I*.

For *Publication III*, a split-root technique was explored to investigate how the phosphorus nutrition status affects the accumulation of rare earth elements (REE) in roots and shoots of plant species that exhibit different P-acquisition strategies. In this study, six plant species (*Triticum aestivum* cv Arabella, *Brassica napus* cv Genie, *Pisum sativum* cv Karina, *Cicer arietinum* cv Kabuli, *Lupinus albus* cv Feodora and *Lupinus cosentinii*) were cultivated on two sand substrates (quartz sand and a mixture of quartz sand (Q) and river sand (M)) Figure 3, the substrate properties are further explained under the substrate characterisation section.

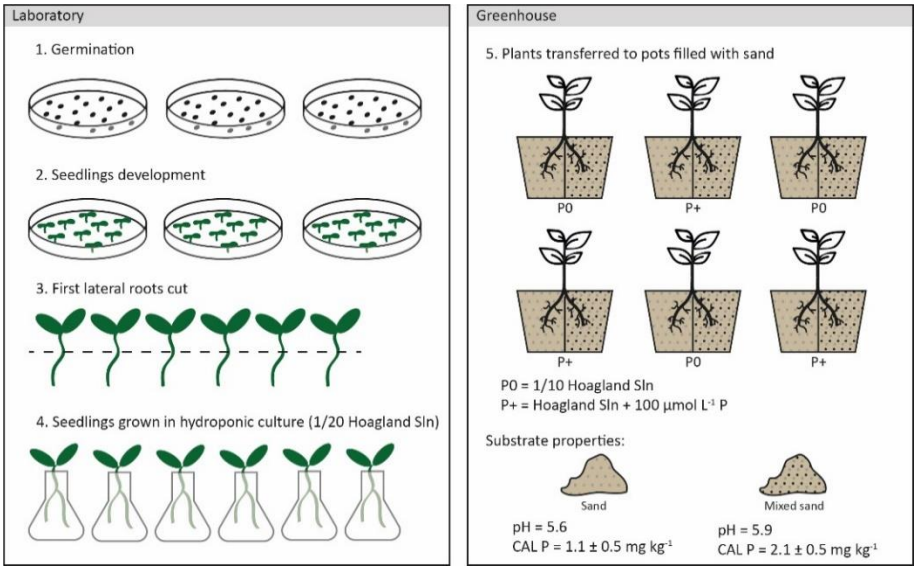

Figure 3 *A demonstration of the experimental setup for the split root experiment using Triticum aestivum, Brassica napus, Pisum sativum, Cicer arietinum, Lupinus albus and Lupinus cosentinii cultivated on quartz sand and a mixture of the quartz sand with river sand.*

In *Manuscript I*, we investigated the relationship and interactions between essential and non-essential elements in plants with different nutritional strategies and silicon absorption capacities. The experiment was a semi-hydroponic experiment, where a peat substrate was used. The five species cultivated were rape seed, (*Brassica napus* L. cv Genie), white lupin (*Lupinus albus* L. cv Feodora), pea (*Pisum sativum* L. cv Karina), maize (*Zea mays* L. cv. Badischer Gelber) and cucumber (*Cucumis sativus* L. cv Paksa). Two weeks after germination, the plant species were first treated with the Hoagland solution, prepared according to Hoagland and Arnon (1950), and the contents are presented in Table 2. Four weeks after germination, the respective treatments were applied, represented in Table 3. The treatments applied were aluminum (Al), rare earth elements (La, Nd, Ce, Gd and Er, further denoted, REE), and Al+REE. Furthermore, the mixture of Al+Cd+REE (Trace elements, further denoted TE) was applied as a differentiation from the plants treated with TE and Silicon (TE+Si) at concentrations of 10 µmol L^{-1} and

100 µmol L^{-1}, respectively, for each element. Silicon was applied at 1.5 mmol L^{-1}. The Hoagland solution was prepared, with the components and applied in a 1:5 strength as represented in Table 2.

Table 2 *Treatment Solutions administered and the corresponding species.*

Concentrations Hoagland Stock solution	Concentration Stocksolution [g/L]	Amount of stock solution/ 1 Litre
1M NH_4NO_3	80	1
2M KNO_3	202	2.5
2M $Ca(NO_3)_2 \cdot 4H_2O$	236/0,5	2.5
1 M KH_2PO_4	136	1
2M $MgSO_4 \cdot 7H_2O$	493	1
H_3BO_3	2.86	1
Fe-EDDHA	5	1.5
$MnCl_2 \cdot 4H_2O$	1.81	1
$ZnSO_4 \cdot 7H_2O$	0.22	1
$CuSO_4 \cdot 5H_2O$	0.051	1
$H_2MoO_4 \cdot 2\ H_2O$	0.09	1

Table 3 *The experimental structure for Manuscript I.*

Treatment	Elements	Treatment Concentration [µmol L^{-1}]	Plant species	Replications
AL	Al	10 µmol L^{-1} 100 µmol L^{-1}	*B. napus* *L. albus* *P. sativum* *Z. mays*	3
REE	La, Ce, Nd, Er and Gd	10 µmol L^{-1} 100 µmol L^{-1}	*B. napus* *L. albus* *P. sativum* *Z. mays*	4
Al + REE	Al, La, Ce, Nd, Er, Gd	10 µmol L^{-1} 100 µmol L^{-1}	*B. napus* *L. albus* *Z. mays*	4
Trace elements (TE)	Al, Cd, La, Ce, Nd, Er, Gd	10 µmol L^{-1} 100 µmol L^{-1}	*B. napus* *C. sativus* *L. albus* *P. sativum* *Z. mays*	5
Trace elements (TE + Si)	Al, Cd, La, Ce, Nd, Er, Gd, Si	10 µmol L^{-1} 100 µmol L^{-1}	*B. napus* *C. sativus* *L. albus* *P. sativum* *Z. mays*	5

3.2 Plant Analysis

The plant biomass harvested to complete the studies from (*Publications I, II and III* and the Manuscripts) were all harvested at 1cm above the soil. The harvested biomass was dried at 60 °C for 24 hours, and there after ground to fine powder and further stirred in centrifuge in preparation for microwave digestion. The microwave digestion (Ethos plus 2, MLS) for all plant species was conducted according to the method by (Krachler et al., 2002). Firstly, a 100 mg subsample of the plant material was weighed in digestion tubes. The weighed samples were then moistened with 0.2 ml of deionised water, then 1.9 ml of 65% nitric acid (HNO_3) was added for sample processing. This process was left to react up to four hours. Additionally, CELERY, NCS ZC73032, used as certified reference material, and a blank sample, were digested similar to the plant samples as reference and control. Subsequently, 0.6 ml of 4.8% hydrofluoric acid was added, and the tubes were inserted into the microwave segments. The tubes were heated to 200 °C for 25 minutes, kept at a constant temperature for 5 minutes, and then cooled down for 30 minutes until the samples reached 75 °C. For inductively coupled plasma mass spectrometry (ICP-MS), in a 1:10 dilution ration, the digested solution was diluted with deionised water and 100 µL rhodium-rhenium internal standard solution was added.

3.3 Soil Characterisation and Analysis

To characterise and determine the distribution of the elements in all the substrates used for the *Publications I, III and IV*, as well as Manuscript I sequential extraction was applied following the method in Wiche et al. (2017). The extraction process follows six fractional steps undertaken in sequence. The first fraction involves shaking the sample in 1 M ammonium acetate (at pH 7) for 24 hours to determine exchangeable elements. In Fraction 2, acid-soluble elements are determined by shaking the sample with ammonium acetate (pH 5). Following this is Fraction 3, where elements that are bound to organics are determined by heating the sample with continuous addition of small quantities of H_2O_2 until a total volume of 10 ml is reached. Fraction 4 involves the isolation of non-crystalline components (Fe, Mn and Al- oxides) using 0.2 M ammonium oxalate (pH 3.2) by selective dissolution. Fraction 5 is mainly for the dissolution of crystalline components (Fe and Al-sesquioxides) where samples were shaken after being mixed with 0.2 M ammonium oxalate and 0.1 M ascorbic acid. Each step was followed by a collection of supernatants from the centrifuged sample and later analysed by ICP-MS for determining the concentration of Ca, Cd, Al, Si , Mn and REEs (La, Nd, Ce, Gd and Er) represented in *Publication I* (Table 1), *Publication III* (Table 1) and *Manuscript I* (Table 1), *Publication IV* (Table 4).

The soil samples were further characterised, where 50 g samples were collected randomly from the bulk and dried in an oven at 60 °C for 24 hours. A sample size of 10g from the dried sample was soaked and shaken in 100 ml of deionised water to determine the pH, specific electrical conductivity and cation exchange capacity (CEC). The respective parameters were measured using the Seven Excellence Multiparameter electrode, InLab® 731-ISM electrode, and the Expert InLab® Pro-ISM electrode, which are all from METTLER TOLEDO.

The cation exchange capacity was determined using a barium chloride ($BaCl_2$) solution, where 1 g of sample from the dried substrate was soaked with 30 ml 1 M $BaCl_2$ and further shaken for one hour. The mixture was centrifuged at 10 000 rpm for 10 minutes to decant the supernatant. This step was repeated three times to ensure that barium was

fully attached to all possible exchangeable binding sides on the substrate. The sample was further soaked in 0.25 M $BaCl_2$, where it was then decanted and then mixed with 30 ml from 0.02 M $MgSO_4$ solution. The mixed samples were shaken overnight, followed by centrifuging and decantation. The supernatant was collected, and element concentrations were determined through inductive coupled plasma-mass spectrometry (ICP-MS, XSeries 2, Thermo Scientific).

3.4 Statistical Methods

In this thesis the main elements of interest were concentrations of Ca, P, Fe, Mn, Si, Al, Cd and REEs. The concentrations of rare earth elements were classified as the sum of light rare earth elements LREEs (La, Ce, Nd) and heavy rare earth elements HREEs (Gd, Er), with the groups separated according to (Tyler, 2004). All elements were measured from the shoots of *H. vulgare* (leaves and tillers), *L. albus* and *L. angustifolius* (*Publication I*), the full shoot biomass of *B. napus*, *C. sativus*, *L. albus*, *P. sativum* and *Z. mays* (*Publication II*), and for *Publication III* from the shoots of *Triticum aestivum*, *Brassica napus*, *Pisum sativum*, *Cicer arietinum*, *Lupinus albus*, and *Lupinus cosentinii*. The statistical calculations were applied for plant yield, concentrations and contents (calculated as concentration × biomass) in all the experiments.

In *Publications I* and *III*, a t-test with Bonferroni adjustment of p-values was used to compare significant differences between means of element concentrations in soil fractions, carboxylate concentrations of P+ and P0 plants, and element concentrations in plant parts cultivated with different P-supply. For the one-way ANOVA, Duncan's post hoc test was used for significant effects ($p < 0.05$). Additionally, Multivariate analysis of variance (MANOVA) using a type III model was used to assess the means of plant yield, element concentrations and contents in various plant parts from both the monocultures and mixed cultures with different lupins (*Publication I*). In *Publication II*, Bartlett's test was used to verify the homogeneity of variances preceding the analysis. In *Publication II*, Fishers LSD post-hoc test (LSD-test) was used to indicate significant effects between the different treatments and the reference plant and between low and high concentrations of the treatments. In *Publications III*, one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was used to compare the different groups. In cases where the ANOVA assumptions (homogeneity) were violated, the data were log+1 transformed. If the assumptions were still violated, significant differences between means were identified using Welch's ANOVA at $\alpha = 5\%$. All statistical calculations were conducted using IBM SPSS Statistics 25. The software R Studio (R-Tools Technology Inc. 2020) was used for visualisation with the ggplot2 package (Wickham, 2016).

4 Results

This chapter focuses on the results obtained from the laboratory and field experiments conducted within the scope of this work to understand how REE bioavailability and uptake in plants is influenced. This chapter aims to highlights of the results obtained from the Publications and Manuscripts. The complete data (graphs and tables) are fully described and discussed in the appended publications. The main points elaborated in the results focus on the uptake of rare earth elements and nutrients, being influenced by substrate properties, mixed culture systems and P-supply, carboxylate release as a response to P-supply and P-deficiency, microorganism activity, the application of silicon fertilizer, as well as the interaction of REEs with other elements such Al and Cd.

4.1 Plant Nutrition and Growth as Affected by Substrate Properties

In *Publication I* the plants were cultivated on Substrate A (pH = 7.9) and Substrate B (pH = 6.8). MANOVA results showed that the substrate significantly influenced nutrient concentrations, P ($p < 0.1$), Ca, Fe, and Mn (all $p < 0.001$), Table 3, *Publication I*. Leaf concentrations on Substrate B were 13% (P), 45% (Ca), 213% (Mn) and 44% (Fe) higher than those on Substrate A. In the same manner, stem concentrations of plants cultivated on Substrate B were 43% (P), 31% (Ca) and 220% (Mn) higher than those on Substrate A. In terms of P treatment application, the biomass of *H. vulgare* was higher in plants treated with NK (1. 5 g P m^{-2}) than NPK (3 g P m^{-2}) by 195%. Intercropping with *L. angustifolius* and *L. albus* and applying P fertiliser led to higher P, Ca, Mn and Fe in *H. vulgare* cultivated on Substrate B than on Substrate A. Furthermore, mixed cultures with *L. angustifolius* increased Ca in *H. vulgare* when cultivated on Substrate A.

The shoot contents were calculated from the biomass and respective element shoot concentrations. MANOVA calculations determined that the effects of the substrate on contents of the micro and macronutrients were intertwined with culture types that were used and the P-treatment (Table 4, p.32). In this case, in the plants cultivated on Substrate A, shoot P, Fe, and Mn contents increased in the mixed cultures under low P-supply compared to the monocultures. In the contrary, in Substrate B, shoot P and Mn contents decreased with no changes in Fe, compared to the barley monocultures (Figure 1, *Publication I*).

4.2 Effects of Substrate Properties, Intercropping and P-supply on REE Concentrations and Contents

The experiments conducted in *Publication I* aimed at exploring the effects of substrate properties and P treatment on REE uptake when intercropping *H. vulgare* with *L. albus* and *L. angustifolius*. Considering REE concentrations, the growth substrate strongly affected REE shoot concentrations and had a more strongly pronounced effect on LREE ($p < 0.001$) than on HREE ($p = 0.05$), Table 4, *Publication I*.

Although P treatment had no significant influence on the concentrations of REEs in the mixed cultures in Substrate B, in barley monocultures there was a decrease in shoot LREE and HREE concentrations in Substrate A. Interestingly, the effect of high P treatment was observed in the mixed cultures with *L. angustifolius*, indicating a significant increase in LREE and HREE in the shoots of *H. vulgare*. Unfortunately, in this study (*Publication I*) *L. albus* was solely cultivated on the two substrates with higher dosing of P fertiliser, thus,

further evaluation of the responses in mixed cultures to different P availabilities is not possible.

In terms of REE contents (calculated from the biomass and concentrations) (Figure 4), the contents of REEs were highly influenced by the substrate they were cultivated on ($p < 0.01$, Table 4, *Publication I*), also showing higher REE contents, which were higher in Substrate A than Substrate B. Intercropping with *L. albus* and cultivating the plants on Substrate B led to decreased LREE and HREE in the shoots of barley, compared to the barley monocultures when treated with NPK (3 g P m^{-2}). These effects were not observed when cultivating on Substrate A, showing that P-treatment and mixed cultures with *L. albus* had no effect. Since *L. angustifolius* was only cultivated on plants treated with NK, the effects of intercropping and P application could not be assessed, but generally mixed cultures with *L. angustifolius* led to a decrease in barley shoot LREE (44%) and HREE contents in Substrate B, Figure 4.

Table 4 *Multivariate ANOVA based on shoot contents (µg m^{-2}) of barley plants exploring for effects of the growth substrate, fertiliser addition (3 g m^{-2} P or 1.5 g m^{-2} P, respectively) and culture form (mono and mixed cultures).*

Plant tissue	Source of variation	P	Ca	Mn	Fe	LREE	HREE
Shoots	Substrate	(*)	(*)	***	(*)	*	(*)
	Fertiliser	NS	NS	NS	NS	NS	NS
	Culture	NS	NS	NS	NS	NS	NS
	Substrate*Culture	**	*	**	(*)	**	**
	Fertiliser*Culture	NS	NS	NS	NS	NS	NS
	Substrate*Fertiliser*Culture	*	NS	*	*	NS	NS

(*) $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; NS not significant.

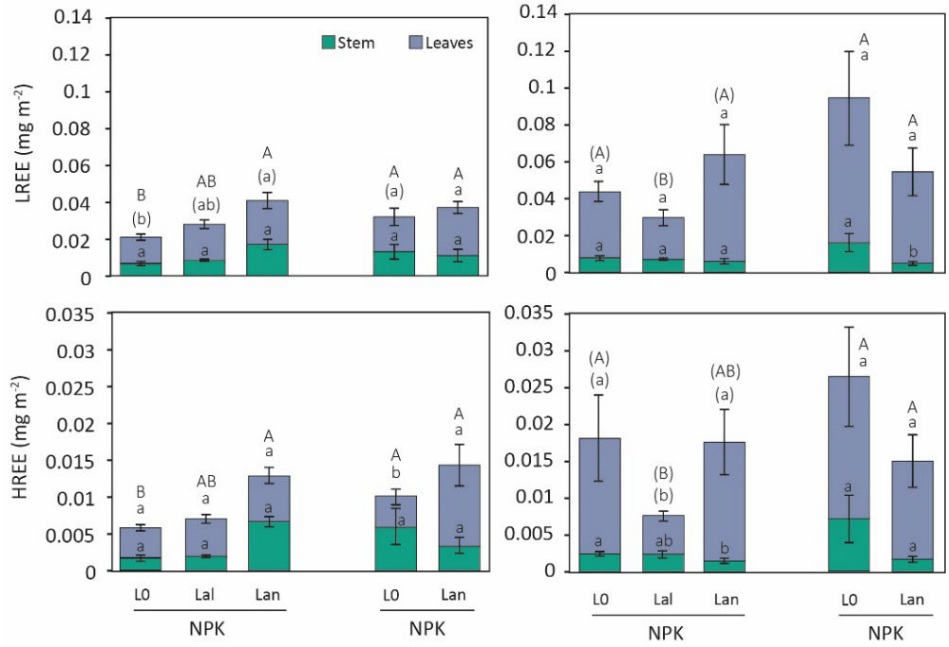

Figure 4 *Total LREE and HREE uptake in stems, leaves and shoots (represented by total height of bars) accumulated in barley monocultures (L0) and mixed cultures with white lupine (Lan) or narrow leaf lupine (Lal) on Substrate A and Substrate B. Representing mean ± sd, for 5 replicates. Significant differences calculated with MANOVA, and Duncan's post-hoc test, where small letters represent differences between the monocultures and mixed cultures, and capital letters differences between substrates and P-treatment (Publication I).*

4.3 Carboxylate Release in Response to P-supply

A laboratory experiment was designed to investigate carboxylate release in *Publication I* when *L. albus* and *L. angustifolius* were supplied with P-treatment, low P (20 µmol L^{-1}) and high P (200 µmol L^{-1}). The results demonstrated that the main carboxylates released were citrate and malate, which at higher concentrations compared to fumarate which was at times below detection limit (as shown in Table 2 in *Publication I*). Under low P-supply *L. albus* released high citrate whereas malate did not change. On the contrary, when P was supplied at 200 µmol L^{-1}, *L. angustifolius* responded with increased malate (224% and 243%, respectively) per unit of dry mass, which were higher than at low P-supply. The two species did not differ in terms if carboxylate release under low P-supply. However, increasing the P-supply showed that *L. angustifolius* released significantly high concentrations of exudates, up to 1100% citrate and 140% malate ($p < 0.05$), compared to *L. albus*.

Another carboxylate analysis experiment was conducted in *Publication III*, however in the study 6 species were cultivated in a split-root experiment to evaluate carboxylate release in P treated *B. napus*, *T. aestivum*, *P. sativum*, *C. arietinum*, *L. albus* and *L. cosentinii*. The results demonstrated that similar to *Publication I*, the main carboxylates released were also citrate and malate, Figure 5. Generally, the species reacted differently to P deficiency according to their nutrient strategy. At low P-supply *Brassica napus* and *P. sativum* responded with a decrease in carboxylate release by 20% and 44%, respectively

(Figure 5A). In contrast, the reduction of P-supply significantly increased total carboxylate release in the lupines, by 159% ($p < 0.01$, *L. albus*) and 115% ($p = 0.03$, *L. cosentinii*), showing an increase of both malate and citrate. Furthermore, in the split roots, carboxylate release per unit time (Figure 5B), was higher in the roots cultivated in mixed sand treated with low P-supply (P0) than in quartz sand in all species. The addition of P-supply (P+) led to increased carboxylate release per unit time in *B. napus* and *C. arietinum*, cultivated on quartz sand. On the contrary, in *B. napus*, *L. albus* and *L. cosentinii* carboxylate release decreased when cultivated on quartz sand (Figure 5).

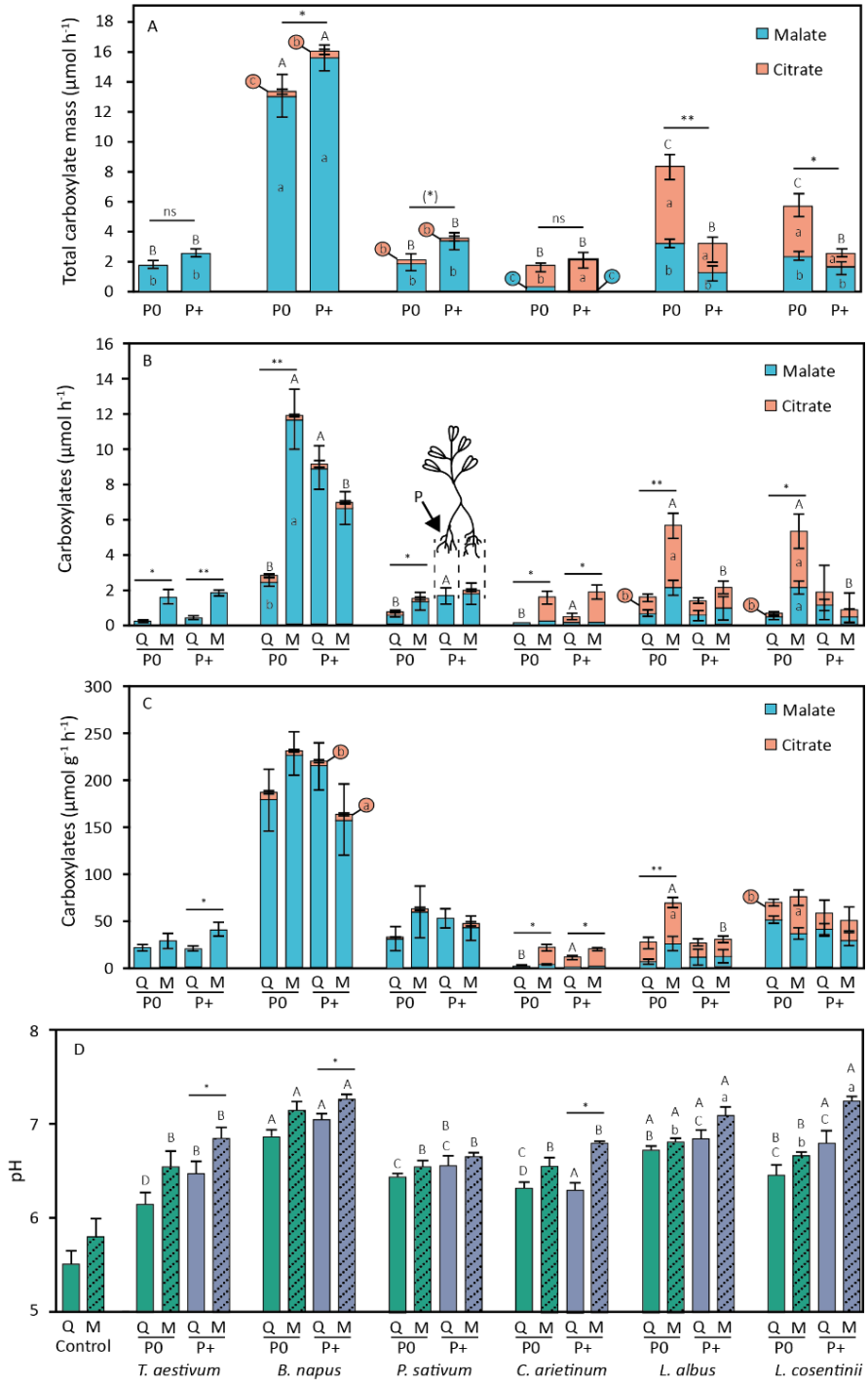

Figure 5 *A representation of carboxylate release in plants (A) Total carboxylate release per plant, (B) carboxylate release from root halves growing in quartz sand (Q) and mixed sand (M) (µmol h^{-1}), (C) exudation rates (µmol g^{-1} h^{-1}) from the different root halves, and (D) rhizosphere pH depending on treatment of plants with 100 µmol L^{-1} P (P+) or no P (P0) from the root half growing in quartz sand (Publication III).*

4.4 Effects of P-supply on Element Uptake in Split-Root Experiment

Brassica napus had highest shoot Cd concentrations compared to *T. aestivum*, *P. sativum*, *C. arietinum*, *L. albus*, and *L. cosentinii*. *Pisum sativum* had highest REE concentrations compared to all the other species. However, *B. napus*, *C. arietinum* and *L. albus* had higher HREE than to *T. aestivum* and *L. cosentinii*. When treated with P, low P treated *L. albus* had lower Cd compared to the plants treated with the high P treatment. Additionally, LREE and HREE concentrations decreased in *L. albus* and *P. sativum* (42% and 44%, respectively) when treated with low P-supply. Under P-deficient conditions, LREE concentrations in *B. napus* and *T. aestivum* were the highest, but there was no effect on the HREE concentrations due to P-supply (Table 4, *Publication III*).

The shoot element contents several plant species were calculated as biomass multiplied by the respective element concentration, represented in Figure 6, *Publication III*. *Brassica napus* had the highest Cd, Al, and REE contents, especially when supplied with P, while Triticum aestivum had the lowest content. Low P-supply did not significantly affect the shoot REE content of some species, but in *B. napus*, LREE and HREE contents tended to be lower. In *L. albus* and *P. sativum*, LREE and HREE contents were significantly lower at low P-supply, and Al and Cd contents were also lower in *L. albus*. However, Al and Cd contents in *P. sativum*, *C. arietinum*, and *L. cosentinii* were largely unaffected by P-supply, Figure 6.

The effects of P-supply (low P and high P, 200 µmol L^{-1}) were also assessed on roots split into two different substrates, quartz sand and mixed quartz sand. In all the plant species cultivated (*B. napus*, *T. aestivum*, *P. sativum*, *C. arietinum*, *L. albus*, and *L. cosentinii*) LREE and HREE concentrations were higher in roots growing on the quartz sand than on the mixed sand, irrespective of the P treatment applied, Figure 6. Aluminium was higher in *B. napus* and Cd concentrations higher in *P. sativum* when treated with low P, cultivated on quartz sand compared to mixed sand. In quartz sand, P-supply had no influence on Al, Cd, and REE in the roots of *T. aestivum*, *C. arietinum*, *L. albus* and *L. cosentinii*. *Pisum sativum* responded with increased Cd and decreased HREE root concentrations, but no effect is seen on Al and LREE when exposed to P-deficient conditions.

The concentrations of Al in the roots of all species were not affected by sand type or P-supply. In *B. napus*, Cd, LREE, and HREE concentrations were significantly higher at low P-supply. In *P. sativum*, the concentrations of all elements were not affected by P-supply. In *T. aestivum*, Cd concentrations were not changed, while LREE and HREE concentrations were lower at low P-supply. The LREE/HREE ratios were highest in *L. albus* and *C. arietinum* and lowest in *L. cosentinii*. The ratios were higher in roots grown in quartz sand than those in mixed sand, except for *T. aestivum* and *P. sativum*. In *T. aestivum*, the ratios were higher in roots grown in mixed sand of P-supplied plants than in corresponding roots grown in quartz sand. Adding high P to the quartz sand decreased the ratio in *P. sativum*. Similarly, in *L. albus*, P addition decreased the LREE/HREE.

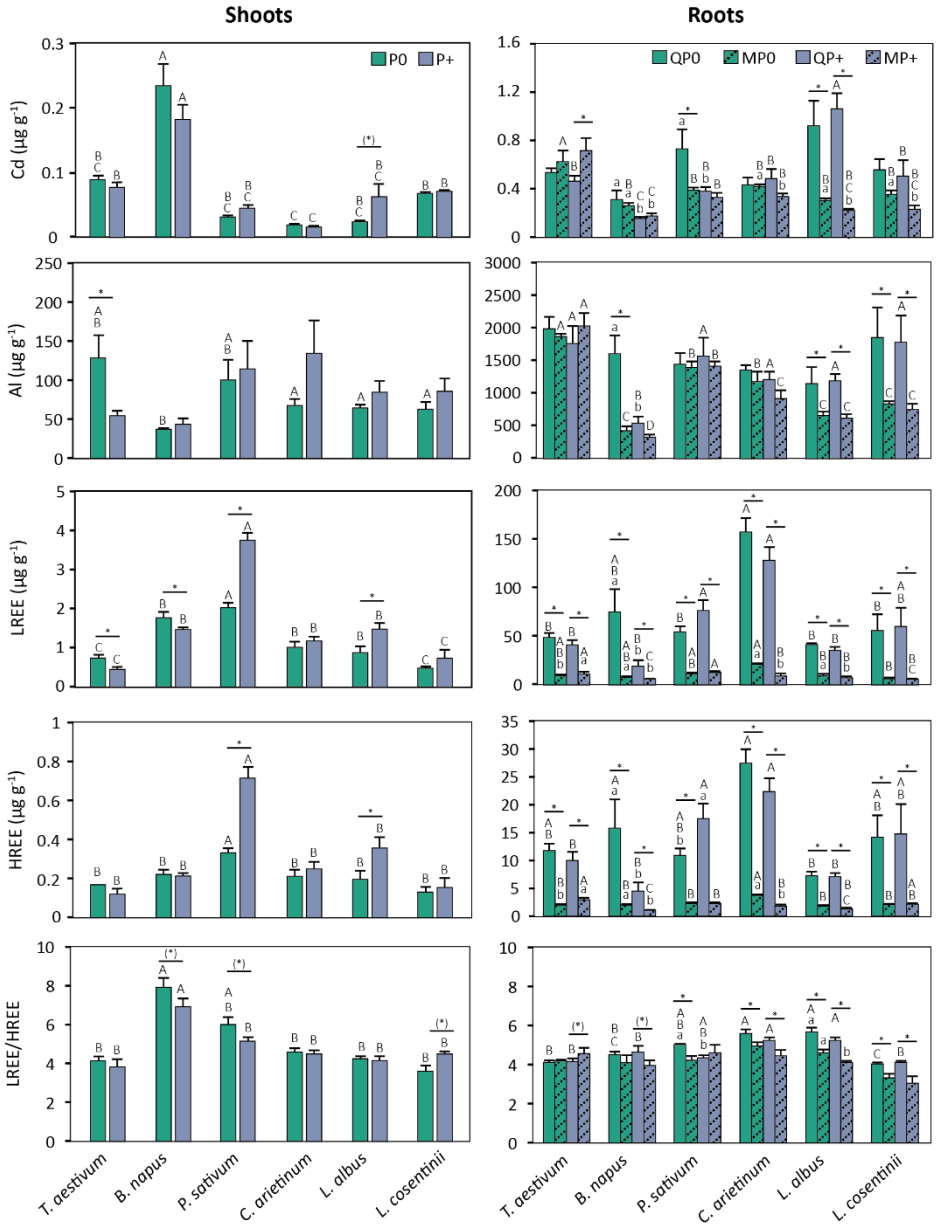

Figure 6 *Root and shoot concentrations of split-root plants treated without phosphorus (Low P, P0) or with 100 µmol L^{-1} P (P+) at the root half growing in quartz sand and mixed sand. Means ± sd, n = 5. Asterisks in shoots indicate significant differences between P-treatments, while averages with the same capital letters across plant species within a P-treatment were not statistically different at α = 5%. Differentiation between plant species within a certain root half and P-treatment is represented by capital letters. Differences between P-treatments on the root side and within a species are represented by lowercase letters. Asterisks also show significant changes between root sides within a certain P-treatment at p < 0.05 in the case of roots (Publication III).*

4.5 The effects of *B. amyloliquefaciens* on the Accumulation of Elements

In *Publication IV*, the effects of soil inoculation using plant growth promoting bacteria (PGPR) *B. amyloliquefaciens* were investigated on *F. esculentum* and *Z. mays*. Inoculating the soil with PGPR led to increased biomass yield for *F. esculentum* (8%) and *Z. mays* (18%), compared to the reference plants(Figure 2, *Publication IV*). Interestingly the application of PFPR did not influence nutrient uptake in *Z. mays*. The *F. esculentum* plants cultivated on soils inoculated with PGPR, Fe contents decreased by 15%, and Ca increased by 40% (Figure 3, *Publication IV*). Furthermore, the application of *B. amyloliquefaciens* did not influence REE uptake in *Z. mays*, but significantly increased in *F. esculentum* (Figure 4, *Publication IV*).

4.6 The Effects of Silicon Treatment on the Uptake of Nutrients and REEs in Different Species

4.6.1 Element Concentrations in Plants as Affected by Silicon Treatment

The plants treated with a mixture of Al, Cd and REE (TE) without silicon had no influence and did not change the biomass of *L. albus* and *B. napus* at 10 µg g^{-1}TE. However, *C. sativus* responded with decreased biomass at both TE treatment concentrations (by 48% and 49% at 10 and 100 µg g^{-1}, respectively) (Table 4, *Manuscript I*) while *Z. mays* only had increased biomass at high TE treatment with up to 40%. The plants showed different responses when it comes to nutrient uptake when treated with TE. *Brassica napus* responded with increased Ca and P only at low TE treatment, but at high TE Only Ca increased. *Lupinus albus* had increased Ca, P, Mn and Fe when treated with low TE, but at high TE only Ca, P and Mn increased. *Cucumis sativus* responded with increased Ca and decreased Fe, while there was no effect on Si, P and Mn. However, at high TE supply *C. sativus* had significantly increased Ca, P and Mn. In *Z. mays*, only Ca increased (by 200%) while other elements were not influenced by low TE application, and high TE application only increased P and Si, (Table 4, *Manuscript I*).

In the same light the application of low TE led to increased LREE and HREE in all species, where LREE concentrations were higher than HREE in all species, compared to the reference plants. Light REE and HREE concentrations increased in *B. napus*, *C. sativus*, *L. albus* and *Z. mays* significantly when TE+Si was applied. At high TE a similar trend was also observed, where all REEs increased in all species Table 5. The application of high TE+Si (100 µg g^{-1}) significantly increased the concentration of both LREE and HREE in all species, compared to the reference plants, however, there were no significant differences between TE and TE+Si in *B. napus* and *L. albus*, Table 5. The highest REE concentrations were found in *C. sativus* when treated with TE+Si, especially at high doses. Similar to other TE and TE+Si treatments, the concentrations of LREE were higher than HREE in all species.

Table 5 *The results of the greenhouse experiment representing concentrations of LREE and HREE in plants treated with TE (Al, Cd and REE) and TE+Si (Al, Cd, REE with Si) varying treatment concentrations between 10 µmol L^{-1} and 100 µmol L^{-1}. Data represent mean ± sd (5 replicates). Small letters indicate statistical difference between each treatment with the reference plants within the same species at α = 5%, (Manuscript I).*

Species	Treatment	Cd (µg g^{-1})	LREE (µg g^{-1})	HREE (µg g^{-1})
B. napus	Reference	0.3 ± 0.1c	0.18 ± 0.09c	0.04 ± 0.02b
	TE(10)	7.9 ± 4.6bB	2.1 ± 1.3b	1.4 ± 0.9b
	TE+Si(10)	4.3 ± 0.5bB	0.89 ± 0.41c	0.6 ± 0.3b
	TE(100)	24 ± 7aA	17 ± 13a	12 ± 8a
	TE+Si(100)	38 ± 12aA	12 ± 4.8a	10 ± 4a
L. albus	Reference	0.11 ± 0.04b	0.05 ± 0.01c	0.01 ± 0.004c
	TE(10)	0.17 ± 0.12B	0.6 ± 0.2b	0.4 ± 0.1b
	TE+Si(10)	0.06 ± 0.04B	0.5 ± 0.3b	0.3 ± 0.2b
	TE(100)	2.3 ± 0.82bA	8 ± 3aA	6.8 ± 2.5a
	TE+Si(100)	4.3 ± 3.0aA	8 ± 2aA	5.4 ± 2.7a
C. sativus	Reference	0.07 ± 0.01c	0.10 ± 0.02d	0.03 ± 0.01d
	TE(10)	1.0 ± 0.24aB	1.8 ± 1.1c	0.8 ± 0.3c
	TE+Si(10)	0.5 ± 0.1bB	0.7 ± 0.3d	0.5 ± 0.3c
	TE(100)	5.8 ± 1.3bA	6.3 ± 3.9b	5 ± 3b
	TE+Si(100)	9.5 ± 1.1aA	22 ± 5a	18 ± 5a
Z. mays	Reference	0.02 ± 0.01e	0.08 ± 0.04d	0.02 ± 0.004c
	TE(10)	6.0 ± 1.8cB	1.2 ± 0.4a	0.8 ± 0.2b
	TE+Si(10)	3.8 ± 1.5dB	0.4 ± 0.1b	0.2 ± 0.1b
	TE(100)	13 ± 3.1bA	2.9 ± 1.6b	2.2 ± 1.3b
	TE+Si(100)	24 ± 5.6aA	6.9 ± 1.3a	5.6 ± 1.4a

4.6.2 Element Contents as Affected by Silicon Treatment

The study evaluated the effect of a treatment containing aluminium, cadmium, and rare earth elements (TE) at 10 µmol L^{-1} and 100 µmol L^{-1} on the uptake of Cd, Al and REE in four plant species. At a dose of 10 µmol L^{-1}, TE increased the uptake of Cd and REE in all species, indicating higher LREE than HREEs in the plants. The addition of TE at 100 µmol L^{-1}, also increased the uptake of Cd, LREE and HREE in all species, with *B. napus* having the highest LREE and HREE compared to *L. albus* and the Si-accumulators *Z. mays* and *C. sativus*.

The inclusion of Si in the TE (Al, Cd and REE) treatment resulted in an increase in Cd uptake across all species, as observed in Figure 7. Additionally, the uptake of REE increased in *B. napus* and *C. sativus* when treated with high doses of TE+Si. Notably, *C. sativus* showed the highest uptake of LREE (67 µg), while *B. napus* showed the highest uptake of HREE (37 µg). Compared to the reference plants and the TE treated plants, all species demonstrated higher LREE and HREE uptake when treated with high TE+Si. On the other hand, the introduction of Si to low TE concentrations led to a 2-fold increase in Al uptake in *B. napus* and *Z. mays*, while no significant differences were observed between TE at 10 and 100 µmol L^{-1}. Meanwhile, there was no significant difference between 100 µmol L^{-1} TE and TE+Si in terms of Al uptake.

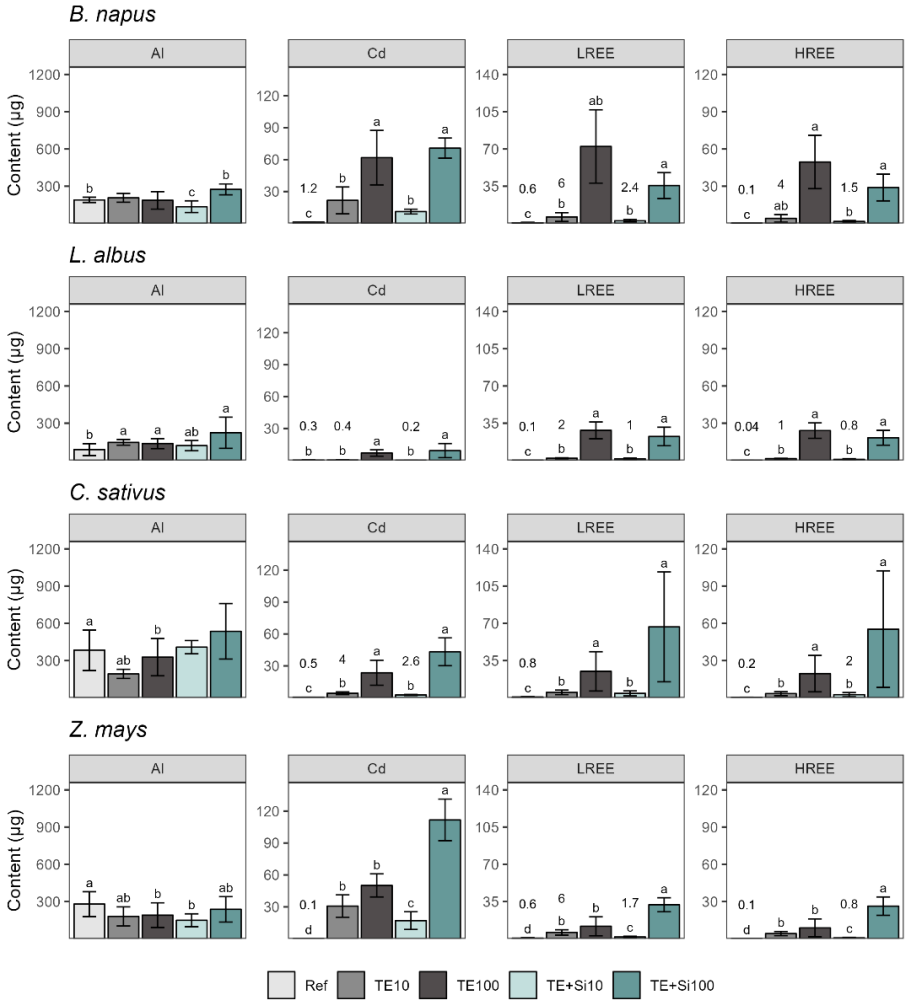

Figure 7 *Total uptake of Al, Cd, LREE and HREE in in plants treated with TE (Al, Cd and REE) and TE+Si (Al, Cd, REE with Si) varying treatment concentrations between 10 µmol L^{-1} and 100 µmol L^{-1}, Ref = reference. The values are means of 5 replicates and error bars show standard deviation. Small letters indicate statistically significant differences between each treatment within the same species at α = 5% (Manuscript I).*

5 Discussion

In this thesis the focus is placed on the rhizosphere activities that may affect phytomining of REEs. The experiments conducted in *Publications I*, *III*, and *IV* as well as *Manuscripts I* serve as a contribution towards the understanding of plant-soil processes associated with the phytomining of rare earth elements (REEs) utilising potential bioenergy crops with different nutritional strategies. The application of phytoextraction fully depends on the bioavailability of elements in the soil, which makes it imperative to understand plant-soil interactions, element interactions in the soil solution, as well as plant adaptation towards rhizosphere chemical changes. Generally, not all elements present in the soil occur in plant-available forms, necessitating plant adaptation and changes in the rhizosphere to increase the possibility of REE uptake.

This chapter forms the synthesis of all the findings from the different experiments conducted. To understand the factors influencing the efficiency of the phytomining of REEs, it is important to understand how soil properties (mainly soil pH), the application of phosphorus treatment, and intercropping influences REE uptake in plants. Additionally, the effects of silicon (Si) on the accumulation of rare earth elements, and the chances of REE co-accumulation with other elements such as Cd and Al. Lastly, the preferential accumulation of REE in plants and their distribution in plant organs, must be understood as they are relevant for determining which plants to harvest in phytomining.

5.1 The Relationship Between Substrate Properties, P-Supply and Intercropping on REE Uptake

5.1.1 Effects of Substrate Properties and Application of P on REE Uptake in Plants

Substrate properties influence nutrient availability in plants, which can be used as an indicator for plant growth and development (Kovarikova et al., 2019). Furthermore, it is widely accepted that similar to other elements, REE availability, mobility, speciation and uptake are greatly influenced by the interaction between the plants and the substrate, especially in the rhizosphere (Pourret et al., 2019).

The field experiment in *Publication I* varied substrate properties, evaluating how the soil properties, especially pH would influence the availability and uptake of nutrients and REEs. Substrate A was characterised with a relatively high pH (7.9), while Substrate B had a relatively lower pH (6.8). The results showed that higher shoot nutrient concentrations (P, Ca, Fe, and Mn) were observed in monocultures of *H. vulgare* cultivated on Substrate B (pH = 6.6) compared to those cultivated on Substrate A (pH = 7.9). The barley plants showed nutrient deficiency, in terms of Mn and P ($P < 2$ mg g^{-1} and $Mn < 50$ µg g^{-1}) when the plants were treated with 1.5 g P m^{-2} and cultivated on the more alkaline Substrate A.

The addition of P-fertiliser alone had no effect on P uptake in barley, even when cultivated in mixed cultures with the lupines. This could have occurred because the concentration of the treatment applied was not high enough to influence plant reliance on the treatment as a source of P. The transfer of P from the soil through the roots to the shoots depends highly on the P-status in the soil (El Mazlouzi et al., 2022). Furthermore, fertilisation often is of limited efficiency because roots only absorb a small portion of the applied P, causing a substantial amount of P to remain in the soil as unavailable P (Campos et al., 2018).

From literature, there is an understanding that REEs have similar chemical properties as with other elements, including Ca and Al, and can also be influenced by the presence of P and Fe (W. S. Liu, Guo, et al., 2019; Rengel, 1994; Wiche, Tischler, et al., 2017). This is because a) REEs are chemically similar to nutrients, and b) phosphates, organic matter and Fe-oxyhydroxides play a significant role in the availability of REEs as they act as hosts for these elements (Cao et al., 2001; Tyler, 2004; Tyler & Olsson, 2005; Wiche et al., 2015). Furthermore, the substrate characterisation (Table 1, *Publication I*) using sequential extraction showed that the REEs were mainly available in soil fractions 3–5 (where, F3: elements in oxidizable matter, F4: amorphous oxides and F5: crystalline oxides). At low pH, there are high concentrations of dissolved organic matter, which can influence the solubility and mobility of REEs (Cao et al., 2001; Kovarikova et al., 2019; Tyler, 2004). Compared to alkaline soils, acidic soils typically have higher concentrations of REEs because the desorption on soil particles is reduced, resulting in lower retention of REEs in the soil and higher mobility and bioavailability. (Tyler, 2004). This can be attested by the results from *Publication I*, where LREE and HREE concentrations were lower in the alkaline Substrate A than in the slightly acidic Substrate B, especially when the P- fertiliser was supplied. This suggests that in acidic conditions, the insoluble REE fraction would be displaced from their binding sites on soil minerals and released into the soil solution, making them available to plants (Cao et al., 2002).

In alkaline conditions REEs are said to form complexes with calcium carbonates (Okoroafor, Kunisch, et al., 2022; H. Tang et al., 2016). This is because REEs have a higher valency (3+) compared to Ca (2+), which allows REEs to bind strongly to soil components, including Fe- and Mn-oxides, soil organic matter, or even to surfaces of root cells. When bound to the aforementioned components, REE mobility in the soil solution is highly limited, and as such, not available for plant uptake (Cao et al., 2002; Marsac et al., 2021; Pourret et al., 2019; Wiche et al., 2015). It should also be mentioned that REEs are typically taken by the roots of most plant species as free ions, which are soluble in water. Yet, due to their poor solubility in neutral or alkaline soils, free REE ions cannot be taken up by plants in high quantities (Ramos et al., 2016; Tyler, 2004).

The application of P in REE rich soils is considered effective in immobilising REE through the formation of REE-phosphate complexes, which are then fixed in the soil. This complexation can occur either through precipitation with phosphates or through cation exchange between free K^+ (in KH_2PO_4) and REEs bound to soil particles, making them inaccessible for plant uptake (H. Tang et al., 2016). Turra et al (2019) reported that the application of phosphate fertiliser increased REE concentrations in *Citrus limonia*, indicating high REE concentrations in the leaves compared to branches. This contradicts the results from *Publication I*, where the application of P fertiliser on the barley monocultures decreased REEs significantly, especially on the alkaline Substrate A, while this treatment had no effect on the slightly acidic Substrate B. This is possibly because there were insoluble REE-phosphate precipitates formed, reducing REE accessibility to the plants as reported by Jin et al. (2019).

5.1.2 The Effects of Intercropping and P-Supply on REE Uptake

In intercropping, nutrient acquisition strategies play a significant role in how plants accumulate different elements (Dissanayaka & Wasaki, 2021). Following this, the lupines have a higher nutrient acquisition efficiency (especially P) compared to barley, which explains the high nutrient concentrations in the lupines, as seen in *Publication I*, see also Nobile et al. (2019). Furthermore, intercropping with *L. albus* and *L. angustifolius* and

applying P fertiliser led to higher P, Ca, Mn and Fe (by 13%, 45%, 213% and 43%, respectively) when cultivating on the slightly acidic Substrate B compared to the more alkaline Substrate A. Legumes help intercropped cereals acquire P, primarily in alkaline and neutral soils where rhizosphere acidification in response to N_2 fixation increases P availability (Xue et al., 2016). Linking these effects on the results from substrate properties, it shows that substrate properties and nutrient status (P- status) contribute significantly to successful intercropping (Schwerdtner & Spohn, 2021; Xue et al., 2016).

Two factors in which plants interact with each other in intercropping have been highlighted, namely complementarity and interspecific facilitation (Schwerdtner & Spohn, 2021). In complementarity, competition for resources between intercropped species is reduced, as plants take up nutrients or other elements through separate mechanisms, at different rates or from separate fractions of the soil (Dissanayaka & Wasaki, 2021; Xue et al., 2016). Resource facilitation, on the other hand, refers to mutually beneficial interspecific relations between plant species, where resource allocation and rhizosphere conditions are improved to benefit both intercropped species (L. Li et al., 2007; Xue et al., 2016). These interspecific root relations between intercropped plants have also been reported to improve plant biomass yield, and the acquisition of macro and micronutrients (Ca, Fe, Mn, and P) as well as non-essential trace elements such as Cd, in plants growing in their proximity (Cu et al., 2005; Egle et al., 2003; L. Li et al., 2021; L. Li et al., 2004; Muler et al., 2014; Wiche, Székely, et al., 2016). Possible mechanisms involved when intercropping for interspecific resource facilitation include: a) rhizosphere acidification, which increases the solubility and availability of inorganic P compounds such as $FePO_4$, $AlPO_4$ and even $REEPO_4$ and b) carboxylate release (as a result of P-deficiency), resulting in the chelation of Fe and REEs, further increasing the mobility and availability of P to neighbouring plants, also summarised in Figure 8,p.45 (C. Liu et al., 2022; Zhou et al., 2009).

From what is observed in Figure 8, it is possible that while intercropping can be beneficial for the mobilisation of micro and macronutrients as well as micronutrients such as Fe and Mn, REE availability can also be influenced, especially considering the P-status in the soil (Honvault et al., 2021). Interestingly, in *Publication I*, although REE uptake was mostly influenced by substrate properties independent of the mixed cultures and P-supply, intercropping with *L. albus* led to a significant decrease in REE accumulation, especially at the application of high P. Thus, in these mixed culture plots, the high P present in the substrate can immobilise REEs. On the contrary, high P- supply (P+) on the mixed cultures between *H. vulgare* and *L. angustifolius* led to increased REE concentrations especially under alkaline conditions. This can be attributed to carboxylate release in *L. angustifolius* as a result of high bicarbonate (further explained in section 5.1.3).

These results highlight that under P abundant conditions, intercropping with *L. albus* would decrease REE uptake in the neighbouring species, limiting the available REEs to the root system of *L. albus*. However, mixed cultures with *L. angustifolius* under high P-supply led to high REE concentrations in *H. vulgare* shoots. Intercropping can therefore be expected to yield or extend positive effects towards the uptake not only of nutrients but also of REEs when the nutritional status (especially P) in the soil is moderate or low (Lambers et al., 2015).

5.1.3 Accumulation of REEs Due to Carboxylate Release and P-Supply

Although P, Mn and Fe are present in the soil, plants usually do not readily access them, which can be a limiting factor towards plant growth as they are also essential for plant growth. This is because P and Fe are found primarily in complex and semi-insoluble compounds in the soil, making them not readily available (Wiche, Székely, et al., 2016). In such cases plants, adapt to such conditions through altering their root morphology, and physiological processes (Lambers et al., 2013; Pavlovic et al., 2013). Increased root surface accompanied by the development of lateral roots, root hairs in the apical zone, and transfer cells are among the root morphological alterations (Pavlovic et al., 2013). Additionally, plants respond with the release of carboxylates, protons and siderophores, which in turn assist plants in nutrient acquisition (Lambers et al., 2015; Xing Wang et al., 2013).

In a controlled experiment in *Publication I*, *Lupinus albus* and *Lupinus angustifolius* were studied, to evaluate how their root morphology and the release of carboxylates in response to P-deficiency change and how these changes can influence nutrient and REE accumulation. This experiment was conducted on the basis that lupine species generally respond with the formation of cluster roots and the release of citrate and malate when exposed to P-deficient conditions (Lambers et al., 2013; Lambers et al., 2015; Xing Wang et al., 2013; Wiche, Székely, et al., 2016). To explore this phenomenon further, *Hordeum vulgare*, reported to have an inefficiency towards P-acquisition (Wiche, Székely, et al., 2016) was intercropped with the two lupines to evaluate how the P-acquisition strategies exhibited by *L. albus* and *L. angustifolius* would influence not only the uptake of P and other nutrients, but also of REEs in *H. vulgare*.

The results reveal that *H. vulgare* accumulated high concentrations of Ca, Mn and Fe in mixed cultures with the lupines, when exposed to low P-supply, which can be attributed to the effective nutrient efficiency of the lupines. Furthermore, the results showed that the mixed cultures had a significant influence on REE uptake, which can be attributed to the release of carboxylates in lupines (Table 2, *Publication I*). This demonstrates that the release of carboxylates to promote P-acquisition cannot be limited to a specific element, but can influence other elements too, such as REEs which are always associated with the elements such as P, Mn, and Fe, and are similar in terms of chemical properties (Pearse et al., 2007; Wiche, Székely, et al., 2016). It is worth noting that the carboxylate experiment for the lupines was conducted in a controlled greenhouse experiment, to have a clear picture of how the plants would respond to P-supply. This was necessary because root samples cannot be liberated from field soils without damaging them. Damaging the roots would lead to obtaining unreliable results (Tyler, 2004). The results obtained were then used to infer the performance of the lupines in terms of carboxylate release, as well as to further explain the interactions between carboxylate release and the uptake of elements in neighbouring species.

Furthermore, since carboxylate release was reduced in the barley with increased P-supply (Table 2, *Publication I*), it is possible that the fraction of REEs that was available was only limited to the root system of the lupines. A such, REEs would not be made available to the neighbouring barley plants. A reduced carboxylate release when REEs have formed REE-phosphate complexes with P would implicate that the REEs remain immobile and inaccessible for plant uptake (C. Liu et al., 2021). Ding et al. (2005) also reported that the complexation of REEs with P (especially at high P conditions) to form insoluble precipitates in the root cells of *T. aestivum* significantly contributes towards the restriction of the translocation of REEs from the roots to the shoots.

On the contrary, P-supply on the mixed cultures between *H. vulgare* and *L. angustifolius* led to increased REE concentrations especially under alkaline conditions. This can be attributed to root exudation and proton release in *L. angustifolius*, as affected by the high bicarbonate in the alkaline substrate. *Lupinus angustifolius* is less tolerant towards bicarbonates (usually present in high pH) compared to *L. albus* (W. Ding et al., 2020). The presence of bicarbonates or calcareous conditions inhibits root growth in *L. angustifolius*, which would respond with the release of carboxylates to overcome the stress, inevitably mobilising P-bound REEs for plant uptake (W. Ding et al., 2020). This then would explain why intercropping with *L. angustifolius* led to increased carboxylate release, consequently increasing REE concentrations and content in *H. vulgare*, when P was added (as mentioned in section 5.1.2).

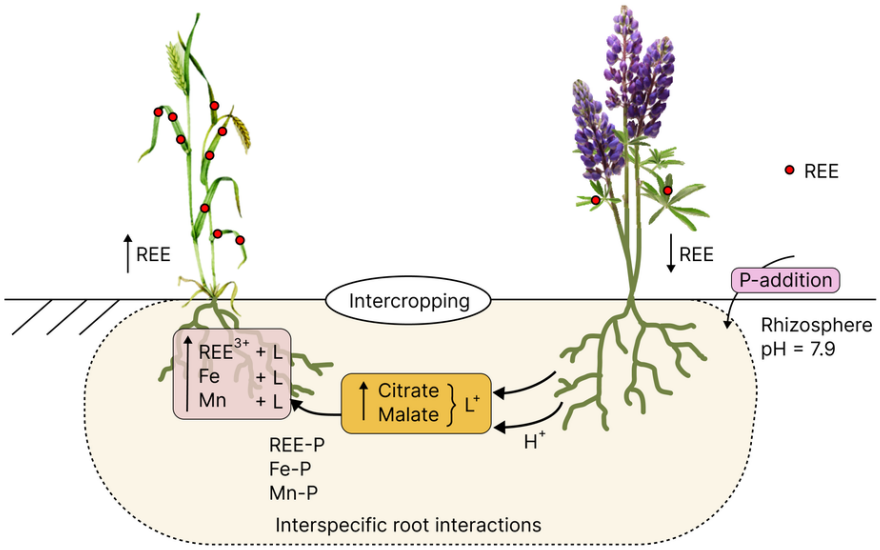

Figure 8 *Possible ways in which insoluble REEs are liberated from soil particles and taken in up in plants when intercropping. Ligands (L), released from plants (as carboxylates and/or siderophores) and from microorganisms in the rhizosphere. The addition of exogenous P towards an intercropping system with P-efficient species in alkaline soils, can lead to increased release of ligands and protons, which can in turn increase the mobility of REEs, even Fe and Mn. This describes the interspecific interactions between the species, as the REEs are made more available for uptake in the neighbouring species.*

In *Publication III*, the phenomenon of carboxylate release was explored more, observing the direct influence on REE uptake. In *B. napus*, the concentrations of Al, LREE, and HREE did not change under low P, with reduced biomass. Consequently, reduced biomass would lead to low contents of Al, LREE and HREE. It is also emphasised that *B. napus* responded with decreased carboxylate release in the plants exposed to P-deficient conditions. As Ca, K, and Na channels are the primary ionic transporters for REEs, carboxylates and other chelating substances would modify the chemical speciation and subsequently the absorption and accumulation of REE, also influencing the LREE/HREE ratio (Han et al., 2005; Wiche, Tischler, et al., 2017). Furthermore, *B. napus* released high malate. Generally, malate has low complexation constants towards REEs, however since

it has been released in high quantities, complex formation with REEs should be favoured, as well as element exclusion from the roots, hence the decreased REE accumulation (S. Ding et al., 2005; Wiche, Tischler, et al., 2017).

This is supported by a recent study by Liu et al. (2023) which demonstrated how the cultivation of *Phytolacca Americana* (pokeweed) under P-deficient conditions induced the release of root exudates. The increased release of organic acids was reported to accelerate the mobilisation of P and REEs, further increasing plant REE uptake. Additionally, introducing exogeneous carboxylates (more specifically citrate), led to similar results to those of naturally released carboxylates. This highlights the significance of carboxylates in P-mobilisation under limited P conditions. Furthermore, it implies that P-efficient plants apply the upregulation of citrate and malate as a response to P-limitation (C. Liu et al., 2021). It is also important to acknowledge that soil microbes are associated with ligand secretion, thus contributing towards increasing REE mobilisation and bioavailability (C. Liu et al., 2023; Schwabe et al., 2021). These reports significantly correspond to the findings in *Publication III*, where *L. albus* released carboxylate under P-deficient conditions, consequently increasing shoot REE uptake. Furthermore, in *Publication III*, the presence of *B. amyliloquefaciens* led to increased REE accumulation in *F. esculentum* compared to the reference plants (Figure 4, *Publication IV*). This can also be attributed to the organic acids released (carboxylic acids and siderophore) by the bacteria as well as the root exudates from plants which increase the solubility of the elements in the rhizosphere leading to increased uptake (Alemneh et al., 2020).

These findings therefore, suggest that P-acquisition strategies, especially the response with carboxylate release and rhizosphere acidification are inevitably involved in increasing the mobilisation of target elements (in this case REEs) (Lambers, 2022) when plants are undergoing nutrient deficiency stress. This is incongruency with the results obtained in *Publication I*, *Publication III,* and *Publication IV*, as well as those reported by Wiche et al. (2016).

5.2 Co-Accumulation of REEs with Cd and Al influenced by Silicon

The benefits of Si include the ability to alleviate toxicity stress (Cd, Al, Zn) or nutrient deficiency (Fe) from different elements in various plants (J. Ma et al., 2015; Pavlovic et al., 2021; Pavlovic et al., 2013; Ye et al., 2012). Although the toxic effects of Cd and Al have been extensively reported, toxicity of REEs is limited and has no detrimental effects towards plants or the environment (Kovarikova et al., 2019; Tyler, 2004). Plants have developed ways to adapt towards element toxicity by releasing ligands (such as phytosiderophores and organic acids) (Kabata-Pendias, 2010; Peñaloza et al., 2004; Piñeros et al., 2005). The ligands play a significant role towards enhancing root element accumulation, tolerance, and reducing the toxicity of elements such as Al, Cd and Ni. Generally, plants take up Si as monosilicic acid (H_4SiO_4), which is accumulated in plant roots and shoots (W. S. Liu, Zheng, et al., 2019). Furthermore, Si has been reported to form stable complexes with REEs ($REE_3SiO_4^{2+}$), which are more stable than complexes formed with ligands (Akagi, 2013).

In this thesis, the experiment in *Manuscript I* differentiated between treating the plants with trace elements Al, Cd and REE (TE) and TE with Silicon and evaluated how the plants would respond to low trace TE (10 µg g^{-1}) and high (100 µg g^{-1}) TE application, with Si kept constant at 1.5 mmol L^{-1}. The reason the treatment included Al, Cd and REE was to mimic multi-elemental soil conditions where Al, Cd and REE can be present at similar concentrations. This applies especially to soils contaminated as a result of mining activity

(Gwenzi et al., 2018; Q. Li et al., 2020). Several studies have reported the co-accumulation of REEs with Al, Mn and Si, especially in REE hyperaccumulators such as *Dicranopteris linearis* and *Phytolacca americana* (W. S. Liu, Zheng, et al., 2019; W. S. Liu, Zheng, et al., 2021). As a result, this phenomenon was investigated on known Si-accumulators *Zea mays* (maize) and *Cucumis Sativus* (cucumber), as well as *Brassica Napus* (rapeseed, a heavy element accumulator) and *Lupinus albus* (white lupin, an excluder plant).

The results from *Manuscript I* clearly demonstrated that nutrient efficiency plays a significant role towards plant tolerance against toxicity of elements such as Al and Cd. For instance, when the TE treatment with Al, Cd and REE was applied, the plants had significantly increased concentrations of these elements in their shoots. *Brassica napus* and *C. sativus* responded with decreased shoot growth when exposed to TE, possibly due to multi-element intoxication and dysregulation of nutrient homeostasis (Kubier et al., 2019; Page et al., 2006). The same species also had reduced nutrient concentrations as a result of the presence of toxic elements. *Lupinus albus* responded with increased accumulation of the nutrients Ca, P, Fe and Mn, despite the presence of the toxic TE. This can be attributed to the effective nutrient acquisition in *L. albus* (Lambers et al., 2015; Neumann, 2000). The acquisition of nutrients is crucial for plant growth and development, and efficient nutrition enables plants to develop tolerance against toxicity (Sarwar et al., 2010). This also helps plants to tolerate toxicity from the toxic elements, including Al and Cd, thereby mitigating their impact on the plant (Pavlovic et al., 2021; Sarwar et al., 2010). From these results it can therefore be stipulated that although REEs can be beneficial to plants, if co-accumulated with Al and Cd, their benefits towards plants are minimised.

The application of Si expectedly increased Si concentrations and contents in the Si-accumulators, *Z. mays* and *C. sativus*. This effect further increased with increasing TE treatment concentrations added to the growth substrate. Interestingly, *B. napus* and *L. albus* also responded with increased Si concentrations, especially when Si was applied with high TE but with no effect on Si content (Figure 3 in *Manuscript I*). The content of elements in plants is a product of plant biomass and element concentrations, and in most of these plants, Si had no effect on the biomass, hence no influence on element content. It is however possible that for the Si-accumulators, the presence of the toxic elements triggered the upregulation of Si transporters, further increasing Si uptake in their tissues (Bhat et al., 2019; J. Ma et al., 2015).

The application of Si and the influence it may have on the accumulation of essential and non-essential elements appeared to be species-specific, indicating that plant innate response towards metal toxicity also plays a role in how they behave when exposed to Si-rich environments (Liang et al., 2007). For instance, in *L. albus*, the concentrations of nutrients and those of Al, Cd and REEs were not influenced by the presence of Si. On the contrary, the Si-accumulators and *B. napus* responded with decreased concentrations of Cd and REEs when treated with Si and TE at 10 μg g^{-1}. Additionally, nutrient uptake was also not affected by the addition of Si. This is possibly because plants such as *B. napus* can accumulate Si in the roots, but since they lack Si-transporters, the Si is limited to the roots and not transported to the shoots. Furthermore, the Si can form barriers around the roots. This would lead to a restricted movement of cations through the roots, thus preventing root-shoot translocation of Cd, REEs and even nutrients (Jian Liu et al., 2013; W. S. Liu, Zheng, et al., 2019).

The plants treated with high TE treatment and Si also responded differently in terms of nutrient uptake, where the Si-accumulators *C. sativus* and *Z. mays* interestingly responded

with increased Fe and Mn uptake. Two possible reasons are proposed for this cause: a) *Z. mays* released phytosiderophores that led to the mobilisation of Fe in the soil, thus increasing the mobility of Fe, and b) the presence of Si possibly upregulated the acquisition and mobilisation of Fe and Mn, for instance by liberating them from Fe-P/Mn-P complexes, thus increasing their uptake in the Si-accumulators (Da Cunha & do Nascimento, 2009; Hernandez-Apaolaza, 2014; Pavlovic et al., 2013).

Calcium concentrations in *C. sativus* and *Z. mays* differed. The concentration of Ca decreased in *C. sativus*, but a substantially increased in *Z. mays*. It is commonly accepted that REEs are transported via Ca channels in plants. However, the varied physiological reactions to Si supply with regard to nutrient accumulation do not appear to have a significant influence on REE accumulation mediated by Ca transporters since REE concentrations in the growth media were orders of magnitude lower than Ca (Han et al., 2005).

Additionally, Si application with high TE treatment (100 μmol^{-1}) increased Al, Cd and REE in the Si-accumulators. It is possible that Si improved tolerance against metal toxicity, through the chemical modification of the apoplast. This therefore led to an increased release of metal chelating compounds, increasing the mobility of Cd and REEs and their radial transport within the plant (Keller et al., 2015). Another possible mechanism by which Si can promote element transport is by enhancing the formation of inorganic Si-REE or Si-Cd complexes, which could be transported from the roots to the shoots and stored in silicified structures (Guntzer et al., 2012; J.-F. Ma, 2004).

The main processes behind the interaction between Si and REEs, Cd and Al have not been explored in the experiments completed within the scope this thesis but opens room for deepened research to verify whether Si could have formed complexes with Cd and REEs. These results therefore indicate that plant functional properties pay a significant role in terms of the co-accumulation of REEs with other elements such as Cd and Al under the influence of Si – especially Si-accumulators as they are able to benefit significantly from Si application.

5.3 Preferential Uptake of REE in Plants

How individual REEs are distributed in the soil and their different binding states depends highly on the soil type and its associated properties (Ramady, 2008; Tyler, 2004). Many characteristics of plants, particularly those connected to the existence of apoplastic barriers, have an impact on REE root to shoot distribution. Rare earth elements initially encounter apoplastic barriers in the roots as the main restricting factor during transportation to the xylem, which hinders their transfer to other plant organs (Yu et al., 2012). Rare earth element concentrations are usually higher in plant roots compared to other plant organs (Tyler, 2004; Yuan et al., 2017). Owing to this, the order of REE contents in various plant organs is as follows: roots > stems > leaves > flowers > fruit > seeds (Brioschi et al., 2013; S. Ding, Liang, Zhang, Huang, et al., 2006; Gmur & Siebielec, 2022; Ramos et al., 2016). It also corresponds to the findings from *Publication III* where most species had higher REE concentrations in in the roots compared to the shoots (Figure 4, p. 33). The rate of REE absorption from the soil to the roots is substantially higher than the rate of translocation from the roots to the shoots (Hu et al., 2006).

Other studies have reported a different pattern where REE contents follow the decreasing order: leaves > stems > roots > flowers > fruit > seeds. This is especially reported for cereals such as oat and rice (Brioschi et al., 2013; Wiche, Kummer, & Heilmeier, 2016; Wiche, Székely, et al., 2016; Yuan et al., 2017). It is in congruency with

the results in *Publication I*, where the *H. vulgare* plants had higher REE accumulation in the leaves compared to the stems, agreeing that the REE accumulation in the barley reflects patterns in cereals (Kovarikova et al., 2019; Wiche, Kummer, & Heilmeier, 2016). This therefore reflects specie-specific mobility of REEs within plants, which can also be influenced by other soil-plant interaction factors (Kovarikova et al., 2019).

Apart from the REE distribution within the plants, the general REE uptake demonstrated higher LREEs than HREEs accumulated in plants (*Publication I*, *Publication II* and *Manuscript I*). In *Manuscript I*, the plants exposed to P-deficient conditions showed increased LREE, especially *B. napus*. Similarly in *Publication I*, *H. vulgare* also had higher LREE accumulated in the plant shoots compared to HREE. A similar trend has been reported in REE accumulating ferns such as *Dryopteris erythrosora*, *Athyrium filix-femina* and *A. niponicum* (Grosjean et al., 2020). This pattern of REEs in plants reflects the natural abundance of the REE in the soil (Tyler, 2004). This can be attributed to two things: 1) preferential uptake of LREEs compared to HREEs, further translocating them in the plant shoots (Grosjean et al., 2019), and 2) HREEs tend to form insoluble complexes with low molecular weight organic acids (LMOWAs), in this case possibly citrate (Grosjean et al., 2020; Grosjean et al., 2019; Ozaki & Enomoto, 2011). Yuan et al. (2017) demonstrated that the preferential accumulation of LREEs in the leaves of *P. americana* can be linked to oxalic acid, which formed insoluble complexes with HREEs, inhibiting their translocation to the shoots. In this thesis these effects were observed regardless of the treatment regime applied to the plants. Furthermore LREE/HREE ratios can be influenced by the distribution of REEs in the growth substrate (Yuan et al., 2017). From the substrate characterisation in this thesis, all the substrates had higher LREE concentrations than HREEs.

On the contrary, from the split experiments, *L. albus* and *L. cosentinii* (*Publication III*) responded with low LREE concentrations and content when exposed to P-deficient conditions, although they had increased citrate and malate exudated. *Lupinus cosentinii* further had low LREE/HREE ratios, compared to *L. albus*, indicating that *L. cosentinii* accumulated higher HREE than *B. napus*. Since *L. cosentinii* acidifies the rhizosphere, the formation of carboxylic acids is accelerated, which can prohibit the formation of HREE complexes in the soil. This therefore means HREE are liberated in the soil and not bound to the organic acids (W. Ding et al., 2020; Pearse et al., 2007). Ding et al. (2006) reported that root to shoot REE translocation inclined more to a prominent fractionation toward HREE, indicating that REE complexation with organic compounds in the xylem could have been involved in the translocation of REEs in wheat.

6 Conclusions

This thesis focused on rhizosphere processes and element interactions affecting the bioavailability of rare elements in phytomining. The results provided insight into how REE mobility and retention in the soil are influenced by their interactions with soil particles and organic substances such as root exudates. The chemical characteristics of the organic molecules and REEs, and soil pH, have an impact on the strength of these bonds formed with REEs. Therefore, these conclusions are made:

1. The results from this thesis demonstrated that substrate properties, especial soil pH and fractions in which REEs are available has a significant influence on REE availability and accumulation towards plants. Plants cultivated on soils with acidic pH had higher REEs accumulated, compared to alkaline soils.
2. High P-supply reduces the availability of REEs towards plants, possibly through formation of insoluble REE-P complexes leading to decreased REE accumulation in plants.
3. The mobilisation of REEs in the rhizosphere of lupines and their transport to neighbouring plants depends on the species-specific ability to respond to different levels of phosphorus supply with carboxylate release, while also influenced by substrate properties such as soil pH.
    - Intercropping with *L. albus* under similar conditions led to decreased accumulation of REEs in the neighbouring species, indicating decreased carboxylate release, which led to the adsorption of the REEs on the roots of the *L. albus* and therefore reducing availability in the neighbouring species. This would be useful in mixed cultures when plants are cultivated on agricultural soils rich in REEs to stabilize the REEs in the soil, to avoid phytotoxicity.
    - Intercropping with *L. angustifolius* on alkaline soils that are characterised with low REE availability and supplying the plants with P leads to increased mobility and availability of REEs for uptake in neighbouring plants. This can be a powerful tool for the phytomining of REEs.
4. Plants employ carboxylate release as a strategy to access P in P-deficient conditions, as a result, they mobilise other trace elements including REEs. Such a strategy includes rhizosphere acidification (through the release of H^+ alongside carboxylates), enabling the formation of soluble REE-ligand complexes available for plants uptake. Additionally, carboxylates can interact with other elements in the soil, such as aluminium and iron, to release bound REEs.
5. Microorganisms also play a significant role in terms of the release of organic ligands that mobilise REEs and thus making them available towards plants. Soil inoculation using *B. amyloliquefaciens* possibly facilitated the solubilisation of REEs in the soil, making them more available for uptake by *Fagopyrum esculentum* (buckwheat).
6. The supplementation of Si reduced the accumulation of Cd and REE when available at moderate concentrations. However, when available under high Al and Cd stress, Si enhanced the accumulation of REEs and Cd in the shoots of Si-accumulators *Z. mays* and *C. sativus*. This indicated that Si plays a significant role in the alleviation of metal toxicity, while promoting coaccumulation of these elements in Si-accumulators.

7. In terms of the application of phytomining and phytoremediation it is crucial to understand in which plant organs are REEs accumulated, since in some plants REE accumulation is high in the roots compared to the above-ground plant organs. It therefore would be useful to choose plant species that are able to accumulate high concentrations of REEs in their shoots.

Overall, the most important link between microorganisms and plant root exudates influencing the bioavailability of rare earth elements in plants is still not clear. This necessitates further research into these processes; characterizing the chemical forms in which REEs exist in the rhizosphere as well as their interaction with carboxylates, to fully explain the dynamics of the processes facilitating the bioavailability of REEs in phytomining.

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Abstract
Phytomining of Rare Earth Elements: Dynamics of rhizosphere processes and element interactions in the soil

Most elements available in the soil are, apart from geogenic origin, also a product of anthropogenic activities. Among these elements, there are rare earth elements (REEs), which are not only prevalent in most geologic environments, but may also be found in some lean ores, abandoned mining sites, and agricultural areas. REE became key to many modern industries including chemicals, consumer electronics, clean energy, transportation and in agriculture. Due to increasing demand, REE are of increasing relevance as raw materials. Besides other conventional extraction methods of elements from the soil, phytoextraction, using hyperaccumulator plants, is known as a feasible way to either extract metals from contaminated soils (through a technology called phytoremediation) or to extract economically valuable metals (via phytomining). The concept of phytomining has been applied specifically focusing on other economic elements such as nickel and gold. The application of phytoextraction depends fully on the bioavailability of elements in the soil, which makes it imperative to understand plant-soil interactions, element interactions from when they are absorbed in plants, as well as plant adaptation towards these changes. Generally, not all elements present in the soil are in plant-available forms, necessitating plant adaptation and changes in the rhizosphere to increase the possibility of uptake.

In this thesis, experimental work under field conditions as well as controlled laboratory and greenhouse experiments were conducted with the aim to understand rhizosphere processes and element interactions affecting the bioavailability of rare earth elements in phytomining. The studies focus on: a) the effects of substrate properties and P-supply in mixed culture crops on the accumulation of rare earth elements (*Publication I*), b) the relationship between carboxylate-based nutrient-acquisition strategies, phosphorus-nutritional status, and rare earth element accumulation in plants (*Publication III*), c) the impact of soil inoculation with *Bacillus amyloliquefaciens* FZB42 on the phytoaccumulation of rare earth elements and potentially toxic elements (*Publication IV*), and d) the relationships between essential and non-essential elements in plants with different nutritional strategies and silicon absorption capacities (*Manuscript I*).

Plants cultivated on a slightly acidic (pH = 6.8) substrate accumulated higher concentrations of nutrients and rare earth elements than on an alkaline substrate (pH = 7.9). Cultivating *Hordeum vulgare* in mixed culture crops with *Lupinus angustifolius* on the alkaline substrate and supplying the plants with P-fertilizer, showed that *H. vulgare* accumulated high nutrient and REE concentrations. Conversely, in the mixed cultures with *Lupinus albus* cultivated on the slightly acidic substrate, REE accumulation in *H. vulgare* decreased significantly. This emphasises that interspecific root interactions between species with different P-acquisition strategies in combination with plant nutrient supply influences REE fluxes between the plants. The results also demonstrated that plants that respond to P-deficiency with carboxylate release as way to access sparingly soluble P in the rhizosphere also increase the solubility and mobility of other elements, including REEs. This occurs due to the H+ ions released alongside carboxylates which acidify the rhizosphere. Therefore, this indicates that rhizosphere acidification and P-acquisition strategies positively influence REE bioavailability.

The results also demonstrated that soil inoculation with *Bacillus amyloliquefaciens* FZB42 significantly increased the accumulation of REEs in plants while reducing the accumulation of potentially toxic elements such as cadmium. Similarly, plants with high silicon absorption capacities (Si-accumulators, such as *Zea mays* and *Cucumis sativus*), tend to accumulate high concentrations of essential and non-essential (REEs, Al and Cd) elements in their shoots. This indicates that Si mobilizes REEs in the soil, increasing their uptake in plants. Furthermore, Si increases plant tolerance against multielement toxicity, allowing Si-accumulators to accumulate high concentrations of Al and Cd without any detrimental effects towards plant nutrition and growth.

Overall, the findings from this thesis emphasise that rhizosphere modification has the potential to improve the efficiency of phytomining while mitigating environmental risks associated with toxic element accumulation through phytoremediation. In terms of practical application, the results of this thesis contribute towards sustainable mining practices (remediation and revegetation strategies on post mining and contaminated landscapes) and promoting the restoration of environmental integrity.

Lühikokkuvõte
Haruldaste Muldmetallide Fütokaevandamine: risosfääri ja Geokeemiliste Protsesside Dünaamika Pinnases

Enamik mullas leiduvatest elementidest on peale geogeense päritolu ka antropogeensete tegevuste tulemus. Nende hulgas on haruldased muldmetallid (REE-d), mis on levinud enamikes geoloogilistes keskkondades, kuid võivad esineda ka madalakvaliteedilistes maakides, hüljatud kaevandusaladel ja põllumajandusmaadel. REE-d on muutunud väga olulisteks mitmetes kaasaegsetes tööstusharudes, sealhulgas keemia-, elektroonika-, puhta energia, transpordi- ja põllumajandustööstuses. Nõudluse suurenemise tõttu kuuluvad REE-d kriitiliste toormete hulka. Lisaks tavapärastele elementide eraldamise meetoditele pinnasest on tuntud ka fütoekstraktsioon, mis kasutab hüperakumuleerivaid taimi metallide eemaldamiseks saastunud pinnasest (fütoremediatsioon) või majanduslikult väärtuslike metallide kättesaamiseks biomassi põletamist (fütokaevandamine). Fütokaevandamist on rakendatud mitmete majanduslikult oluliste elementide, nagu nikkel ja kuld, kontsentreerimisel. Fütoekstraktsiooni rakendamine sõltub täielikult elementide biosaadavusest pinnases ning seetõttu on vajalik mõista taimede ja pinnase vastasmõjusid, elementide käitumist nende imendumisel taimedesse ning taimede kohanemist muutustega. Üldiselt ei esine kõik mullas leiduvad elemendid taimedele kättesaadavas vormis, mis nõuab taimede vastavat kohandumist ja muutusi risosfääris, et suurendada imendumise efektiivsust.

Käesolevas doktoritöös viidi läbi eksperimentaalsed uuringud välitingimustes, samuti kontrollitud labori- ja kasvuhoonekatsed, eesmärgiga mõista juurestiku protsesse ja elementidevahelisi seoseid, mis mõjutavad haruldaste muldmetallide biosaadavust fütokaevandamisel. Uurimus keskendus: a) substraadi omaduste ja fosforisisalduse mõjule haruldaste muldmetallide akumuleerumisel segakultuurides (*Publikatsioon I*), b) karboksülaadil põhinevate toitainete omandamise strateegiate, fosfori kättesaadavuse ja haruldaste muldmetallide akumuleerumise seostele taimedes (*Publikatsioon III*), c) mulla *Bacillus amyloliquefaciens* FZB42-ga nakatamise mõjule haruldaste muldmetallide ja potentsiaalselt mürgiste elementidie fütoakumulatsioonis (*Publikatsioon IV*) ning d) oluliste ja mitteoluliste elementide vahelistele seostele erinevate toitumisstrateegiate ja räni akumuleerimisvõimega taimedes (*Käsikiri I*).

Nõrgalt happelisel substraadil (pH = 6,8) kasvatatud taimed omandasid suuremaid toitainete ja haruldaste muldmetallide kontsentratsioone kui aluselisel substraadil (pH = 7,9) kasvatatud taimed. Kultiveerides *Hordeum vulgaret* segakultuuris *Lupinus angustifoliusega* aluselisel substraadil ning varustades taimi fosforväetisega, täheldati, et *H. vulgare* omandas suures koguses toitaineid ja REE-sid. Samas segakultuurina *L. albusega*, mida kasvatati nõrgalt happelisel substraadil, vähenes *H. vulgares* REE-de akumulatsioon oluliselt. See näitab, et liigisisene juurte vastasmõju erinevate fosfori omandamise strateegiatega liikide puhul ja taimede toitainetega varustamine mõjutab REE-de jaotumist taimede vahel. Tulemused näitasid ka, et taimed, mis reageerivad fosfori defitsiidile karboksülaatide vabastamisega, omastamaks raskestilahustuvat fosforit risosfääris, suurendavad ka teiste elementide, REE-de lahustuvust ja liikuvust. See juhtub koos karboksülaatidega vabanevate vesinikioonide tõttu, mis muudavad risosfääri happelisemaks. Seetõttu osfori hankimise strateegiad, mis muudavad risosfääri happelisust, tõstavad ka haruldaste muldmetallide bio-kättesaadavust.

Doktoritöö tulemused näitasid ühtlasi, et mulla inokuleerimine *Bacillus amyloliquefaciens* FZB42-ga suurendas märkimisväärselt REE-de akumuleerumist taimedes ning vähendas

potentsiaalselt toksiliste elementide (nt kaadmium) esinemist mullas. Samamoodi kipuvad taimed, millel on kõrge räni omandamisvõime (räniakumuleerijad, näiteks *Zea mays* ja *Cucumis sativus*), koguma oma lehtedesse kõrgeid nii oluliste kui ka mitteoluliste elementide (REE-d, Al ja Cd) kontsentratsioone. See näitab, et räni mobiliseerib mullas esinevaid haruldasi muldmetalle ja suurendab taimedel toksiliste elementide taluvust.

Kokkuvõttes rõhutavad käesoleva doktoritöö tulemused, et risosfääri mõjutamine võib parandada fütokaevandamise efektiivsust ning vähendada keskkonnariske, mis on seotud toksiliste elementide akumulatsiooniga pinnases(fütoremediatsioon). Töö tulemused aitavad kaasa jätkusuutlike kaevandamistehnoloogiate väljatöötamisele (taastamis strateegiad kaevandamisjärgsetel ja saastunud maastikel) ning looduskeskkonna taastamisele.

Appendix 1

Publication I

Monei, N., Hitch, M., Heim, J., Heilmeier, H., Wiche, O. (2022). Effect of substrate properties and phosphorus supply on facilitating the uptake of rare earth elements (REE) in mixed culture cropping systems of *Hordeum vulgare*, *Lupinus albus* and *Lupinus angustifolius*. Environ Sci Pollut Res, 29, 57172857189. doi: 10.1007/s11356-022-19775-x

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RESEARCH ARTICLE

Effect of substrate properties and phosphorus supply on facilitating the uptake of rare earth elements (REE) in mixed culture cropping systems of *Hordeum vulgare*, *Lupinus albus* and *Lupinus angustifolius*

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Abstract
This study presents how phosphate (P) availability and intercropping may influence the migration of rare earth elements (REEs) in legume–grass associations. In a replacement model, *Hordeum vulgare* was intercropped with 11% *Lupinus albus* and 11% *Lupinus angustifolius*. They were cultivated on two substrates, A (pH = 7.8) and B (pH = 6.6), and treated with 1.5 g P m^{-2} or 3 g P m^{-2}. Simultaneously, a greenhouse experiment was conducted to quantify carboxylate release. There, one group of *L. albus* and *L. angustifolius* was supplied with either 200 µmol L^{-1} P or 20 µmol L^{-1} P. *L. albus* released higher amounts of carboxylates at low P supply than *L. angustifolius*, while *L. angustifolius* showed the opposite response. Plants cultivated on substrate B accumulated substantially higher amounts of nutrients and REE, compared to substrate A. Higher P supply did not influence the leaf and stem P concentrations of *H. vulgare*. Addition of P decreased REE accumulation in barley monocultures on alkaline soil A. However, when *H. vulgare* was cultivated in mixed culture with *L. angustifolius* on alkaline substrate A with high P supply, the accumulation of REE in *H. vulgare* significantly increased. Conversely, on acidic substrate B, intercropping with *L. albus* decreased REE accumulation in *H. vulgare*. Our findings suggest a predominant effect of soil properties on the soil–plant transfer of REEs. However, in plant communities and within a certain soil environment, interspecific root interactions determined by species-specific strategies related to P acquisition in concert with the plant's nutrient supply impact REE fluxes between neighbouring plants.

Keywords Intercropping · Rhizosphere · Rare earth elements · White lupin · Root exudates · Phytoextraction

Introduction

Carboxylates released by plant roots are an important strategy of plants to access sparingly available phosphorus and micronutrients such as Fe and Mn in soil (Cu et al. 2005). Particularly for P, Fe and Mn, the availability is limited by low solubility of the corresponding element-bearing minerals and interactions with other inorganic and organic soil phases. Specifically, in soils, Fe and Mn are present as Fe oxyhydroxides and Mn oxides, characterized by low solubility above a soil pH of 5. Thus, in alkaline soils, the availability of Fe and Mn is limited by their extremely low solubility of the respective oxides and oxyhydroxides (Suda and Makino 2016) whereas phosphate strongly interacts with calcium by the formation of hardly soluble Ca phosphates. Moreover, under acidic soil conditions and below a pH of 7, Fe, Mn and P often behave in a dual way showing steadily increasing solubility of Fe and Mn, whereas fixation/specific sorption of phosphate on Fe oxyhydroxides and aluminium

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hydroxides increases and leads to accumulation of P in acidic soils in sparingly plant available forms.

Plants adapted to these conditions and evolved towards a self-determined influence on the chemical features surrounding their roots to create an environment which allows nutrient acquisition over a wide range of soil (rhizosphere) conditions. Besides mutualistic interactions with bacteria and fungi and alteration of soil physical properties, the most important and commonly explored mechanisms include acidification of the rhizosphere by release of protons and release of element-chelating carbon compounds such as carboxylates (Lambers 2022). The ability for mobilizing P and micronutrients in the rhizosphere varies considerably in dependence on plant species, functional plant groups (Neumann et al. 2000; Lambers et al. 2013, 2015) or even genotypes in a certain species (Krasilnikoff et al. 2003). Forbs in general and legumes in particular are considered to be P-efficient due to a strong ability to acidify the rhizosphere and release large quantities of carboxylates under P and Fe deficiency (Lambers et al. 2013; Nobile et al. 2019), while grasses such as *Avena sativa* and *Hordeum vulgare* are described as P-inefficient (Wang et al. 2013; Faucon et al. 2015; Lambers et al. 2015). Some forbs develop specialized root structures with abundant root hairs (cluster roots) that release large quantities of carboxylates into the rhizosphere and exhibit a highly efficient P mining strategy of which P mining strategies in *Lupinus albus* and species from the family Proteaceae have been the most profoundly studied (Lambers 2022).

Although these processes related to plant nutrition are initially regulated by nutrient deficiency, both strategies must be generally considered non-element-specific with respect to the effects of the chemical processes in the rhizosphere. That means while nutrient deficiency triggers a shift in metabolism towards elevated proton and carboxylate release, the compounds released do not only attack nutrient-bearing soil phases but also alter solubility and mobility of non-essential elements in the soil. In addition to this, they influence their availability as it has been demonstrated for Cd, Pb and rare earth elements (REEs) (Wiche et al. 2016a). Among these elements, REEs are particularly interesting to study because they (i) are present in almost all soils at concentrations comparable to essential plant nutrients; (ii) share chemical similarities to essential nutrients, particularly Ca; (iii) interact with nutrient-bearing soil minerals (phosphates, Fe oxyhydroxides); but (iv) are still not essential to plants nor strongly toxic (Tyler 2004).

More specifically, the REEs comprise a group of 17 elements from the lanthanide series including lanthanum, yttrium (Y) and scandium (Sc) that are abundant in the Earth's crust with concentrations that vary from 66 μg g^{-1} (Ce), 30 μg g^{-1} (La) and 28 μg g^{-1} (Nd) to 0.3 μg g^{-1} (Lu) (McLennan 2001; Kabata-Pendias 2010; Davranche et al. 2017). As a special feature in this group, all 16 REEs exhibit ionic radii similar to Ca^{2+}; however, under most pedological relevant conditions, REEs form + 3 ions (Wyttenbach et al. 1998) which strongly interact with phosphate and other negatively charged soil constituents (Diatloff et al. 1993; Zhimang et al. 2000; Cao et al. 2001; Li et al. 2014). As an exception in this group, Eu and Ce may also occur in the divalent or tetravalent state (Davranche et al. 2017). There are slight but indisputable differences in ionic radii from light REEs (LREEs) to heavy REEs (HREEs), leading to differences in their absorption and complexation behaviour in soil (fractionation). Consequently, this might also influence their movement in soil–plant systems and availability to plants. Previous studies conducted followed the generic laboratory and field approach, where synthetic REEs were introduced to the cultivation area. In other approaches, the cultivated plants were left to grow under natural conditions without any anthropogenic modifications (Cunha et al. 2012). There is general consensus that rhizosphere processes related to plant nutrition not only affect the availability of nutrients but also of non-essential elements such as Pb, Cd (Wenzel 2009) and REEs since these elements can be mobilized through lowering of pH and presence of organic acids (Wiche et al. 2017a). Under field conditions, Wiche et al. (2016a, b) demonstrated that mixed cultures of P-inefficient grasses with P-efficient legumes increase the uptake of REEs in the grasses which was most likely due to mobilization of REEs in the rhizosphere of lupins and movement of the elements between intermingling root systems which suggested that not the physiological mechanisms of uptake are of relevance for the accumulation levels of REEs in *A. sativa* and *H. vulgare*.

In fact, it is generally assumed that uptake of REE^{3+} ions is mediated mainly, but not solely by Ca^{2+}, Na^{+} and K^{+} channels (Han et al. 2005; Brioschi et al. 2013; Wiche et al. 2017b). Thus, it seems that lupins are able to attack REE-containing soil phases through the release of protons and the exudation of organic acid anions, which renders these elements available for the P-inefficient grasses (Wiche et al. 2016b).

In the present study, we conducted a mixed culture study under field conditions where we cultivated *H. vulgare* (barley), a P-inefficient cereal in the presence of 11% lupins using either *L. albus*, a cluster root-forming legume (white lupin), and *Lupinus angustifolius*, a non-cluster root-forming lupin (narrow leaf lupin). Each of these cultivation forms was set up on two different soils with different soil pH values and thus differences in plant-available nutrients and REEs. Additionally, on each soil, the plant stands received P fertilizer at a rate of 1.5 g P m^{-1} and 3 g P m^{-1}, respectively, to elucidate effects of P supply and soil properties on REE accumulation in monocultured and mixed cultured barley plants. Moreover, in a greenhouse experiment, we

characterized the root exudate composition of both lupins depending on P supply which will give a hint on the plant's behaviour at different P levels in the field. In combination, this ecologically derived experimental approach allows to explore the effects of soil nutrient availability and species-specific rhizosphere properties on REE dynamics in legume–grass associations which is a relatively unstudied research topic hitherto. Knowing the dynamics of the interaction of lupins and P in the rhizosphere, we hypothesise, firstly, that there is an interaction between P supply and REE accumulation in the plants and, secondly, this pattern should depend on the initial availability of nutrients in the substrates determining the nutritional status of the plants and REE mobility in the substrate. Lastly, the effects should depend on the lupin species and, consequently, on the amount and composition of root exudates interacting with soil phases in the intermingling rhizospheres of barley and lupins.

Material and methods

Field experiment

The experiment was conducted at Bauer Umwelt Business, Hirschfeld (Saxony, Germany), in its off-site recycling and remediation centre. A basin of a total volume of 720 m^3 was filled with two homogeneously sieved top soils both characterized as Luvisols. One half of the basin was filled with soil from a road construction location near Freital, Germany (hereafter referred to as substrate A). The second half was filled with topsoil from a mining-affected area in the vicinity of Freiberg, Germany (hereafter referred to as substrate B). Substrate A was a silty loam with a pH (H_2O) value of 7.9. Substrate B was a silty loam with a pH (H_2O) value of 6.8 (Table 1). A summary of plant-available macronutrient concentrations in the two substrates used for the experiment is shown in Table 1. The elements P, Mg and K were extracted by calcium acetate lactate (CAL) and measured with inductively coupled plasma mass spectrometry (ICP-MS). For analysis of mineral N, NO_3^- and NH_4^+ were extracted from soil samples and photometrically determined according to Bolleter et al. (1961) and Hartley and Asai (1963). NH_4^+ acetate (pH 5) was used for the extraction of exchangeable Ca which was determined through ICP-MS. Total concentrations of REEs, P, Ca, Fe and Mn of the substrate and their concentrations in six operationally defined soil fractions as a result of a sequential extraction according to Wiche et al. (2017a) for soil samples prior to the experiment are shown in Table 2. Both substrates were characterized by similar organic matter contents (LOI), CEC and macronutrient concentrations of N, P, K and Mg (Table 1); however, soil A displayed a significantly higher pH value compared to soil B, indicating differences in element availability. Total concentrations of P, Ca and Fe were significantly higher on substrate A compared to substrate B (Table 2). Also, substrate A contained higher concentrations of P, Ca, Mn and Fe in labile element fractions, especially exchangeable (F1), acid-soluble (F2) and organically bound elements (F3) whereas soil B was characterized by an enrichment of these elements in F4 and F5 (P, Ca, Fe) and F3 (Fe, Mn). In contrast, there were no differences in total concentrations between soils regarding Mn and REEs. The REEs showed no difference in fraction 1 and fraction 2 but showed a substantial enrichment of LREEs in F3 of soil B, leading to a 24% higher labile LREE pool in soil B compared to soil A (Table 2).

Plant cultivation

White lupin (*Lupinus albus* L. cv. Feodora), narrow leaf lupin (*Lupinus angustifolius* L. cv. Sonate) and barley (*Hordeum vulgare* L. cv. Modena) were grown within field conditions in both a monoculture and a mixed culture system on 50 plots with an area of 4 m^2 each (Online Resource 1). To avoid interactions between adjacent plots (e.g. root interactions, water discharge, nutrients, REE, and trace metals), a 0.5-m buffer zone was maintained surrounding each plot without vegetation. On each of the experimental plots, the plants were planted in rows (leaving 20 cm between rows) with a total density of 400 seeds m^{-2}. Mixed barley cultures were obtained from the monocultures by replacement of 11% barley plants with the equivalent proportion of individuals of white lupin and narrow leaf lupin, and thus plant densities were equivalent in both barley monocultures (hereinafter referred to

Table 1 Characteristics of the two different substrates used in the semi-field experiment and initial nutrient concentrations at the beginning of the experiment

Sample	pH (H_2O)	LOI%	CEC_{eff} (cmol kg^{-1})	N_{min} (mg kg^{-1}) (dw)	P_{CAL}	K	Mg
Soil A	7.9 ± 0.4	7.8 ± 1.2	15.6 ± 2.3	47 ± 17	23 ± 9	462 ± 137	243 ± 89
Soil B	6.8 ± 0.3	6.4 ± 1.3	14.0 ± 3.0	32 ± 9	34 ± 6	284 ± 66	170 ± 78

The values are means of 20 replicates for each soil (means ± SD)

LOI loss of ignition, CEC_{eff} effective cation exchange capacity, N_{min} mineral N, P_{CAL} calcium acetate/lactate extractable phosphate

Table 2 Total concentration and sequential extraction results (μg g^{-1} dw) for the identification of the total concentrations of trace elements in the soil substrates

Fraction	P	Ca	Mn	Fe	LREE	HREE	LREE/HREE
Substrate A							
Total	1009 ± 213a	12,292 ± 4595a	977 ± 280	31,087 ± 21,848a	109 ± 27	34 ± 7.7	3.2 ± 0.37
F1	31 ± 16a	4526 ± 1526a	77 ± 25a	3.52 ± 1.06a	0.3 ± 0.08	0.1 ± 0.03	0.3 ± 0.03
F2	57 ± 12	1078 ± 436a	194 ± 35a	222 ± 74.3	3.7 ± 0.7	1.4 ± 0.3	0.4 ± 0.02
F3	133 ± 164	409 ± 214a	112 ± 48b	780 ± 1033b	7.6 ± 5.0b	2.2 ± 0.2b	0.6 ± 0.7a
F4	1121 ± 400a	75 ± 24	42 ± 35	6508 ± 2231b	11 ± 4.5	2.5 ± 1.2	0.2 ± 0.04
F5	73 ± 23a	212 ± 80.1	29 ± 12	4756 ± 1203b	3.3 ± 0.8	0.8 ± 0.2a	0.3 ± 0.03
Substrate B							
Total	878 ± 236b	5775 ± 1619b	887 ± 250	25,296 ± 21,848b	106 ± 19	34 ± 6.6	3.1 ± 0.23
F1	20 ± 13b	2955 ± 882b	47 ± 12b	2.5 ± 0.8b	0.3 ± 0.05	0.09 ± 0.01	0.3 ± 0.03
F2	50 ± 21	513 ± 239b	118 ± 30b	181 ± 85.7	3.2 ± 0.7	1.0 ± 0.2	0.3 ± 0.02
F3	169 ± 135	243 ± 79.2b	198 ± 27a	1401 ± 930a	9.4 ± 3.2a	2.6 ± 0.8a	0.3 ± 0.15b
F4	1496 ± 412b	69 ± 20	38 ± 18	8049 ± 1777a	11 ± 4.0	2.3 ± 0.8	0.1 ± 0.01
F5	110 ± 21b	237 ± 84.7	31 ± 5.4	6396 ± 557a	3.0 ± 0.6	0.7 ± 0.1b	0.3 ± 0.02

Given are means ± SD ($n = 10$). Concentrations within the same element fraction between the substrates were compared by *t* tests with Bonferroni adjustment. Means with different letters are statistically significantly different at $\alpha = 5\%$

F1 exchangeable elements, *F2* acid-soluble elements, *F3* elements in oxidizable matter, *F4* amorphous oxides, *F5* crystalline oxides (Wiche et al. 2017b)

as L0 plots) and mixed cultures (hereinafter referred to as Lal and Lan plots, respectively) (Online Resource 1).

Eight days after seed germination and plant development had taken place, the first dose of fertilizer was given to all plants. Each substrate plot with barley monocultures and mixed cultures with white and narrow leaf lupin (Lal and Lan) was dosed with 10 g of N m^{-2} as NH_4NO_3, 11.6 g of K m^{-2} as KNO_3, 3 g of P m^{-2} as KH_2PO_4 and 1.5 g of Mg m^{-2} as $MgSO_4$, representing the fully fertilized reference plants (NPK). Accordingly, each substrate plot of barley monocultures (L0) and mixed cultures with narrow leaf lupin (Lan) received a similar fertilizer composition regarding N, K and Mg but with one half of P (1.5 g of P m^{-2} as KH_2PO_4) representing the low-dosed variant (NK). To ensure consistency in the provision of nutrients throughout the entire experiment and to avert N deficiency (e.g. by leaching nitrate), the abovementioned fertilizer was applied in two separate doses at the beginning of the experiment and a second time 4 weeks later.

Each of the different treatments, including culture forms and fertilizer treatment, was fivefold replicated on each of the two substrates, and within each substrate, the treatments were set up in a fully randomized design. After 8 weeks of plant growth, shoots of barley in monocultures and mixed cultures were cut 3 cm above the soil surface when harvesting. Only the shoots of the inner square meter of each plot were further processed for chemical analysis.

Quantification of carboxylate release

A separate greenhouse experiment was designed for the determination of root exudates in both *L. albus* and *L. angustifolius* depending on P supply. Seeds of *L. albus* cv. Feodora and *L. angustifolius* cv. Sonate were surface sterilized by washing the seeds with 0.5% sodium hypochlorite (NaOCl) for 3 min followed by carefully rinsing with deionized water and allowed to germinate in petri dishes in a climate chamber at 20 °C. After germination, the seedlings of each plant species (one seedling per pot) were planted in 10 plastic pots (2 L total volume) filled with acid (HNO_3)-washed quartz sand. The pots were incubated in a greenhouse for 6 weeks with a 15-h photoperiod, temperature of 18–30 °C, relative humidity of 65% and average photosynthetically active photon flux density of 630 μmol m^{-2} s^{-1}. During a time period of 4 weeks, all plants received weekly 200 mL of a modified nutrient solution according to Arnon and Stout (1939), supplying all necessary plant nutrients except phosphorus. Additionally, for each species, one-half of the plants received 200 μmol L^{-1} K_2HPO_4 together with the other nutrients (high P) while the other plants received 20 μmol L^{-1} P (low P references). After a cultivation period of 4 weeks, the mature plants were carefully removed from the sand by washing with tap water and transferred into glass beakers containing 300 mL of a 2.5 μmol L^{-1} $CaCl_2$ solution where they were let to stay for 2 h under a growth lamp and allowed to release carboxylates into the collection solutions.

Immediately after the collection, the resulting solutions were stabilized with 1 mL L^{-1} Micropur to prevent microbial decomposition of carboxylates according to Oburger et al. (2014) and analysed by means of ion chromatography. Thereafter, the shoots and roots were separated, weighed and dried for 24 h at 60 °C.

Analysis of trace element concentrations and carboxylates

The harvested biomass of field grown plants was separated in leaves and stems and dried at 60 °C in an oven for 24 h. The dried biomass was ground to fine powder and stored in centrifuge tubes. Thereafter, microwave digestion (Ethos plus 2, MLS) was carried out with 0.1 g of the subsample taken from the ground biomass measured in duplicates. Samples were mixed with 1.6 mL nitric acid (65% supra) and 0.6 mL hydrofluoric acid (4.9% supra) and heated to 220 °C in the microwave according to Krachler et al. (2002). Concentrations of P, Fe, Mn, Ca, Mg and REEs (Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu) from the diluted digestion solutions and soil solutions were determined by ICP-MS (XSeries 2, Thermo Scientific) using 10 µg L^{-1} rhodium and rhenium as internal standards.

Concentrations of acetate, malonate, fumarate, glutarate, malate and citrate in the collection solutions were determined by ion chromatography equipped with suppressed conductivity detection (ICS-5000, 4-mm system, Thermo Scientific). Inorganic and organic acid anions were separated at 30 °C on an IonPac® AS11-HC column (Thermo Scientific) using gradient elution with sodium hydroxide as eluent and a flow rate of 1.0 mL min^{-1}. The measuring program started with an 8-min isocratic phase and a sodium hydroxide concentration of 1 mmol L^{-1}, followed by the gradient analysis with a continuously increasing sodium hydroxide concentration up to 40 mmol L^{-1} over a period of 35 min. Finally, the column was flushed for 3 min with 50 mmol L^{-1} sodium hydroxide and equilibrated for 10 min with 1 mmol L^{-1} sodium hydroxide.

Data processing and statistical analysis

Concentrations of LREEs and HREEs in the plant and soil samples were calculated according to Tyler (2004) as the sum of La, Ce, Pr, Nd, Pm, Sm and Eu (LREEs) and Gd, Tb, Y, Ho, Er, Yb, Tm and Lu (HREEs). All element concentrations reported were calculated on dry weight basis. Significant differences among means of element concentrations in soil fractions, carboxylate concentrations of high-P- and low-P-treated plants and P concentrations in lupines cultivated with different P supplies were compared by *t* test with Bonferroni adjustment of *p* values using IBM SPSS Statistics 25. Additionally, concentrations and contents in different plant parts of the same plants were compared by a *t* test for non-independent samples at $\alpha = 5\%$. Means of plant yield, element concentrations and contents (calculated as concentrations × biomass) in different plant parts resulting from different culture forms (monocultures and mixed cultures with different lupins) as well as factors contributing to altered plant accumulation were evaluated by multifactor multivariate analysis of variance (MANOVA) using a type III model. In case of significant effects indicated by a significant Wilks' lambda at $p < 0.05$, Duncan's post hoc test was used. Prior to the analysis, the data was checked for homogeneity of variances using Levene's test. In case that the assumption of homogeneity was violated, the data was log transformed. If the assumption was still violated, significant differences of means were identified by using single comparisons of groups of means using Welch's ANOVA at $\alpha = 5\%$.

Results

Root exudate patterns in L. albus and L. angustifolius affected by P supply

Compared to *L. angustifolius*, *L. albus* produced higher shoot (high P [203%], low P [137%]) and root biomass (high P [400%], low P [233%]), irrespective of P supply (Table 3). P supply did not influence the root and shoot dry mass in *L. angustifolius* as well as the root dry mass in *L. albus*. However, the shoot dry mass of *L. albus* responded to differences in P supply showing a reduction by 35% when plants were supplied with low P doses. From the carboxylates measured, only citrate and malate were detectable in all collection solutions (Table 3), while fumarate was only occasionally present. All other carboxylate signals (acetate, lactate, glutarate, malonate) were below their respective detection limits. Under conditions of low P supply, *L. albus* strongly responded by 271% increased rates of citrate release per unit root dry mass and showed a 71% increased release of citrate per plant (Table 3). In this study, P supply did not alter the release of malate by *L. albus*. In contrast, in *L. angustifolius*, P deficiency did not increase the release of carboxylates. Instead, in *L. angustifolius* in adequately P-supplied plants, exudation rates of citrate and malate per unit root dry mass were 224% and 243%, respectively, higher than those in P-deficient plants. Overall, in *L. angustifolius*, this resulted in a 180% higher release of citrate and 650% higher release of malate in P-supplied plants. A comparison of exudation rates and amounts of carboxylate release per unit root dry mass between two lupin species revealed that there was no difference in the exudation rates under low P supply. However, when the plants received high P doses with the treatment solutions, exudation rates of citrate and malate in *L.*

Table 3 Growth parameters and root carboxylates collected from *L. albus* (Lal) and *L. angustifolius* (Lan) that were semi-hydroponically cultivated under P-deficient conditions (20 µM P: low P) or supply of 200 µM P (high P)

Species	P supply	Growth parameter		Release per plant			Release per dry weight		
		Root dw, g	Shoot dw, g	Citrate, µM h^{-1}	Malate, µM h^{-1}	Fumarate, µM h^{-1}	Citrate, µmol (g dw $h^{-1})^{-1}$	Malate, µmol (g dw $h^{-1})^{-1}$	Fumarate, µmol (g dw $h^{-1})^{-1}$
Lal	High P	0.8 ± 0.2	2.3 ± 0.4	0.7 ± 0.1	0.6 ± 0.4	0.02 ± 0.01	0.8 ± 0.1	1.0 ± 0.3	0.08 ± 0.07
	Low P	0.6 ± 0.3	1.5 ± 0.7	1.2 ± 0.1	0.8 ± 0.2	< 0.01	3.0 ± 1.4	1.1 ± 0.6	< 0.02
p value		0.43	0.08	< 0.01	0.24	0.34	0.03	0.91	NA
Lan	High P	0.16 ± 0.13	0.76 ± 0.45	1.4 ± 0.5	0.6 ± 0.3	< 0.01	9.4 ± 4.1	2.4 ± 0.6	< 0.06
	Low P	0.18 ± 0.08	0.59 ± 0.21	0.5 ± 0.3	0.08 ± 0.01	< 0.01	2.9 ± 0.4	0.7 ± 0.3	< 0.06
p value		0.88	0.82	0.06	0.04	NA	0.04	0.01	NA
p value	High P	< 0.01	< 0.01	0.04	0.83	NA	0.02	0.01	NA
	Low P	0.22	0.07	0.04	0.01	NA	0.95	0.43	NA

The values are means ± SD ($n = 4$). Significant differences among parameters within a species and between species and within a specific P treatment were identified by a *t* test with Bonferroni adjustment

NA not available

angustifolius per unit root dry mass were 1100% (citrate) and 140% (malate) higher than those in *L. albus* ($p < 0.05$). Considering the amounts of carboxylates released per plant individual (µM h^{-1}) under P deficiency, *L. albus* released 140% and 900% more citrate and malate, respectively. In contrast, when P supply was high, *L. angustifolius* released 100% more citrate while the release of malate was similar.

Plant growth and nutrient concentrations in monocultured and mixed cultured barley plants

In all experimental units, biomass of *H. vulgare* shoots consisted mostly of stem biomass which, on average, yielded 122% more biomass per unit area than that of leaves (Table 5). Substrate properties, culture form (mixed culture with different mixing ratios of *L. albus* or *L. angustifolius*) and P fertilization did not influence the biomass yields of stems of *H. vulgare* (Tables 4 and 5), and there were no differences in leaf biomasses between substrates. Also, intercropping and P addition did not influence the leaf biomass on substrate B, neither in plant stands with *L. albus*, nor with *L. angustifolius*. However, on substrate A, mixed culture cultivation with *L. angustifolius* slightly increased ($p = 0.09$) the leaf biomass of barley when barley was cultivated at low P application level (NK) (Table 4) showing a 126% higher leaf biomass compared to the monocultures. This increase resulting from intercropping was not observable in NPK-treated plants on substrate A, and thus, leaf biomasses in mixed cultures grown under NK addition were by 195% higher ($p = 0.06$) compared to those in barley plants grown in NPK-treated mixed cultures.

A comparison of concentrations in leaves and stems, respectively, and considering data from both substrates and all culture forms and fertilizer treatments revealed that

Table 4 Multifactor multivariate ANOVA based on leaf and stem concentrations of barley plants exploring for effects of the growth substrate, fertilizer addition (3 g m^{-2} P or 1.5 g m^{-2} P, respectively) and culture form (monocultures and mixed cultures)

Plant tissue	Source of variation	Yield	P	Ca	Mn	Fe	LREE	HREE	L/H
Leaves	Substrate	NS	(*)	***	***	***	**	*	NS
	Fertilizer	(*)	NS	NS	NS	NS	NS	NS	NS
	Culture	*	NS	(*)	**	NS	NS	NS	NS
	Substrate × culture	NS	NS	NS	NS	NS	NS	NS	NS
	Fertilizer × culture	NS	NS	NS	NS	NS	*	*	(*)
Stems	Substrate	NS	**	*	**	NS	NS	NS	NS
	Fertilizer	NS	NA	NA	NS	NS	NS	NS	NS
	Culture	NS	NS	NS	NS	NS	(*)	NS	*
	Substrate × culture	NS	*	NS	NS	*	**	*	NS
	Fertilizer × culture	NS	NS	NS	NS	NS	NS	NS	NS

NS not significant

(*) $p < 0.1$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 5 Yield of leaves and stems and concentrations of phosphorus (P), calcium (Ca), manganese (Mn) and iron (Fe) in the plant parts of *H. vulgare* depending on substrate (slightly alkaline substrate A and slightly acidic substrate B), P addition as fertilizer (NK: 1.5 g m^{-2} P; NPK: 3 g m^{-2} P) and culture form (monoculture: L0, mixed culture with 11% *L. albus* (Lal) and mixed culture with 11% *L. angustifolius* (Lan))

Culture form	Culture	Leaves					Stems				
		Yield, g m^{-2} dw	P, mg g^{-1} dw	Ca, mg g^{-1} dw	Mn, µg g^{-1} dw	Fe, µg g^{-1} dw	Yield, g m^{-2} dw	P, mg g^{-1} dw	Ca, mg g^{-1} dw	Mn, µg g^{-1} dw	Fe, µg g^{-1} dw
Substrate A											
Fertilizer											
NK	L0	85 ± 13b	1.9 ± 0.4*B*	5.6 ± 1.9	13 ± 3*B*	117 ± 36*B*	155 ± 46	1.4 ± 0.5(b)*B*	2.2 ± 0.5	4.5 ± 0.9*B*	25 ± 9*B*
	Lan	192 ± 116a(A)	2.3 ± 0.5	6.4 ± 1.3	16 ± 3*B*	146 ± 41*B*	317 ± 186	2.3 ± 0.6(a)	2.2 ± 0.2	5.7 ± 0.7*B*	28 ± 5
NPK	L0	65 ± 12	2.3 ± 0.4	5.2 ± 0.9(a)	12 ± 2(a)*A*	111 ± 15*A*	192 ± 41	1.3 ± 0.6*B*	2.1 ± 0.4	5.9 ± 3.2*B*	29 ± 7
	Lan	65 ± 17(B)	2.1 ± 0.4	6.9 ± 1.3(b)*A*	14 ± 2(a)*A*	152 ± 55B	153 ± 26	1.9 ± 0.5	2.3 ± 0.6	5.7 ± 0.8*B*	34 ± 6
	Lal	53 ± 12	2.2 ± 0.4	5.3 ± 0.9(a)	24 ± 7(b)	140 ± 32	157 ± 66	1.3 ± 0.7	2.1 ± 0.4	5.6 ± 1.2	41 ± 10
Substrate B											
Fertilizer											
NK	L0	92 ± 41	2.5 ± 0.8*A*	6.8 ± 1.8(b)	41 ± 4*A*	204 ± 66*A*	158 ± 41	2.5 ± 0.4*A*	2.4 ± 0.5	19 ± 4*A*	48 ± 8(A)*A*
	Lan	95 ± 40	2.0 ± 0.2	8.6 ± 1.2(a)	35 ± 13*A*	202 ± 17*A*	123 ± 23	2.7 ± 0.5	2.8 ± 0.8	12 ± 6*A*	36 ± 7
NPK	L0	61 ± 17	2.1 ± 0.6	8.9 ± 4.8	40 ± 23(a)*B*	177 ± 45*B*	164 ± 47	2.1 ± 0.6*A*	2.7 ± 1.2	15 ± 12*A*	28 ± 6(a)(B)
	Lan	77 ± 32	2.3 ± 0.4	9.3 ± 1.5*B*	47 ± 13(a)*B*	196 ± 61*A*	127 ± 65	2.3 ± 0.4	2.9 ± 0.4	17 ± 9*A*	44 ± 11(b)
	Lal	50 ± 14	2.4 ± 0.5	10.4 ± 3.5	101 ± 49b	184 ± 23	135 ± 34	2.2 ± 0.3	3.6 ± 0.8	16 ± 6	31 ± 6(a)

Means ± SD ($n = 5$). Significant differences in yields and concentrations within a plant part and substrate were identified by MANOVA followed by Duncan's post hoc test. Small letters show differences between means of monocultured and mixed cultured barley within a specific substrate and P treatment. Capital letters denote differences of concentrations in barley plants of a specific treatment between P treatments within a substrate. Capital letters in italics show differences of concentrations in barley plants between substrates at $\alpha = 0.05$

concentrations of all investigated elements were consistently higher in leaves compared to the stems, except for P on substrate B. On substrate A, leaf concentrations were 28% (P), 171% (Ca), 196% (Mn) and 316% (Fe) higher than stem concentrations. On substrate B, leaf concentrations were 201% (Ca), 213% (Mn) and 405% (Fe) higher than stem concentrations.

Compared to the reference plants treated with 1.5 g P m^{-2}, the addition of 3 g m^{-2} P did not affect the concentrations of Ca, Fe, Mn and P in leaves and stems, respectively, irrespective of the growth substrate. The growth substrate strongly affected concentrations of Ca, Mn and Fe ($p < 0.01$) and slightly affected P concentrations ($p < 0.1$) in leaves, while in stems, the growth substrate highly affected P, Ca and Mn concentrations with no significant effects on Fe. Specifically, considering all data from mixed culture types (*L. albus* and *L. angustifolius*) and P fertilizer treatments, leaf concentrations on substrate B were 13% (P), 45% (Ca), 213% (Mn) and 44% (Fe) higher than those on substrate A. In the same manner, stem concentrations of plants cultivated on substrate B were 43% (P), 31% (Ca) and 220% (Mn) higher than those on substrate A. Moreover, besides major effects of the substrate, multifactor MANOVA revealed significant effects of intercropping (culture form) on Mn in leaves ($p < 0.001$) and marginally significant effects on Ca ($p = 0.08$), while in the tillers, concentrations of P and Fe exhibited significant substrate–culture interactions, indicating that the effect of culture form depends on the growth substrate. More specifically in both substrates, concentrations of Ca increased by 33% and 26% in leaves of *H. vulgare* when the plants were cultivated in mixed cultures with *L. angustifolius* compared to the monocultures (L0), whereas there was no significant effect from *L. albus*. Additionally, leaf Mn concentrations increased highly significantly ($p < 0.01$) as an effect of mixed culture cropping with *L. albus* by 100% on substrate A and by 153% on substrate B, while the presence of *L. angustifolius* did not influence Mn in mixed cultured barley. In the stems, mixed cultures with *L. angustifolius* increased the P concentration significantly by 64% ($p = 0.06$) compared to the monocultures but this effect was only visible on substrate A. The presence of *L. angustifolius* significantly increased Fe concentrations in tillers of barley by 57%, but this effect was only observable on substrate B. Compared to the leaves, there was no effect of the mixed cultures on Ca and Mn in tillers of mixed cultured barley, and compared to *L. albus*, the presence of *L. angustifolius* led to more substantial changes in mineral element composition of *H. vulgare*, except for Mn which was highly affected by *L. albus*.

Rare earth element concentrations in different plant parts

Considering both substrate types, all culture forms and fertilizer treatments, concentrations of REEs were constantly

Table 6 Concentrations (µg g^{-1} dw) of light rare earth elements (LREEs) and heavy rare earth elements (HREEs) and their ratio (LREEs relative to HREEs) in the plant parts depending on substrate (slightly alkaline substrate A and slightly acidic substrate B), P addition (NK: 1.5 g m^{-2} P; NPK: 3 g m^{-2} P) and culture form (monoculture: L0, mixed culture with 11% *L. albus* (Lal) and mixed culture with 11% *L. angustifolius* (Lan))

	Culture	Leaves			Stems		
		LREE, µg g^{-1} dw	HREE, µg g^{-1} dw	L/H, µg g^{-1} dw	LREE, µg g^{-1} dw	HREE, µg g^{-1} dw	L/H, µg g^{-1} dw
Substrate A							
Fertilizer							
NK	L0	0.44 ± 0.20A*B*	0.12 ± 0.09	4.4 ± 1.5a	0.08 ± 0.04(A)	0.04 ± 0.02(A)	2.5 ± 0.7
	Lan	0.41 ± 0.19	0.24 ± 0.21	2.7 ± 1.3b	0.04 ± 0.03(B)	0.15 ± 0.13	1.4 ± 0.9
NPK	L0	0.23 ± 0.06bB*B*	0.07 ± 0.02b*B*	3.7 ± 0.6	0.04 ± 0.02b(B)*B*	0.02 ± 0.01(b)(B)*B*	3.2 ± 0.2a
	Lan	0.49 ± 0.21a	0.12 ± 0.05a*B*	3.8 ± 0.7	0.13 ± 0.06a(A)*A*	0.06 ± 0.03(a)*A*	2.2 ± 0.3b*B*
	Lal	0.37 ± 0.15ab	0.10 ± 0.06ab	4.0 ± 0.9	0.07 ± 0.04ab	0.03 ± 0.02(ab)	3.2 ± 0.7a
Substrate B							
Fertilizer							
NK	L0	0.77 ± 0.28*A*	0.18 ± 0.07	4.2 ± 0.5A	0.09 ± 0.04	0.04 ± 0.03	3.1 ± 1.3
	Lan	0.58 ± 0.30	0.16 ± 0.09	4.4 ± 0.7	0.04 ± 0.01	0.02 ± 0.01	3.4 ± 0.6
NPK	L0	0.59 ± 0.14*A*	0.25 ± 0.18*A*	3.0 ± 1.2B	0.21 ± 0.19a*A*	0.13 ± 0.11(a)*A*	3.7 ± 2.6
	Lan	0.68 ± 0.31	0.21 ± 0.08*A*	4.1 ± 1.4	0.05 ± 0.01b*B*	0.012 ± 0.004(b)*B*	4.1 ± 1.4*A*
	Lal	0.48 ± 0.13	0.15 ± 0.07	3.4 ± 1.0	0.05 ± 0.01b	0.017 ± 0.007(b)	3.4 ± 1.0

Means ± SD ($n = 5$). Significant differences in yields and concentrations within a plant part and substrate were identified by MANOVA followed by Duncan's post hoc test. Small letters show differences between means of monocultured and mixed cultured barley within a specific substrate and P treatment. Capital letters denote differences of concentrations in barley plants of a specific treatment between P treatments within a substrate. Capital letters in italics show differences of concentrations in barley plants between substrates at $\alpha = 5\%$

higher in leaves compared to those in stems with LREE/HREEs > 1 (Table 6). On substrate A, leaf concentrations were 442% (LREEs) and 140% (HREEs) higher than stem concentrations ($p < 0.01$). Also, the LREE/HREE ratio was 46% higher in leaves than in stems ($p < 0.01$). On substrate B, leaf concentrations were 540% (LREE) and 280% (HREE) higher in leaves than in stems ($p < 0.01$) with very similar LREE/HREE ratio among the two plant compartments. The addition of P fertilizer did not affect the concentrations of REEs directly (Tables 4 and 6). However, there were significant interaction effects between P application and culture form influencing the REE concentrations in the leaves as well as P application × culture interactions influencing the REE concentrations in the stems. Overall, the growth substrate strongly affected REE concentrations in leaves but not those in stems with a more strongly pronounced effect on LREE ($p < 0.01$) than on HREE ($p = 0.05$). Considering data from all mixed culture forms and P fertilizer treatments, leaf concentrations on substrate B were 64% (LREE) and 72% (HREE) higher ($p < 0.05$) than those on substrate A but with similar LREE/HREE ratio. Application of P fertilizer in monoculture significantly decreased LREE concentrations of leaves (by 48%) and LREE and HREE concentrations of stems both by 50% on substrate A, while on substrate B, this effect was not observable. Also, in the mixed cultures, there was no direct effect of P application and there were no differences in element concentrations between mixed cultured plants that received different fertilizers. Moreover, plants that received only 1.5 g m^{-2} P (NK) showed no differences in elemental composition between monocultures and mixed cultures. However, on substrate A, mixed cultures of barley with *L. angustifolius* that were treated with P fertilizer responded by a significant increase in concentrations of LREEs by 113% and HREE by 88% in leaves and 225% (LREE) and 200% (HREE), respectively, in stems compared to the monocultures.

On substrate A, *L. albus* did not alter the mineral composition of the mixed cultured plants, irrespective of the P application. In contrast, on substrate B, NPK-treated mixed cultures with both *L. albus* and *L. angustifolius* significantly decreased the REE concentrations by a factor of 4 in the case of LREEs or even roughly 1 order of magnitude in the case of HREEs. It has to be noticed that these effects were only prevailing on slightly alkaline substrate A when plant stands of barley and mixed cultures of barley and *L. angustifolius* were treated with higher doses of P fertilizer.

Accumulation of nutrients and REEs

Considering the biomass of leaves and stems and the herein quantified element concentrations, amounts of elements in the respective plant tissues and whole shoot contents were calculated (Fig. 1 and Fig. 2). Plant leaves consistently contained significantly ($p < 0.01$) higher amounts of Ca (30%), Mn (44%) and Fe (87%) and especially of LREEs (265%) and HREEs (158%) than stems, except P which predominantly accumulated in plant stems with 78% higher amounts than in leaves. The growth substrate strongly influenced the element contents in leaves showing significantly higher amounts of all investigated elements in leaves of plants cultivated on substrate B compared to substrate A (Table 7). In stems, only contents of P and Mn were influenced by a general substrate effect (Table 7). Considering all P addition levels and culture forms, plants cultivated on substrate B contained 57% (P), 73% (Ca), 251% (Mn) and 97% (Fe) as well as 158% (LREEs) and 145% (HREEs) more of the investigated elements in the leaves. Additionally, the plants showed 43% (P) and 160% (Mn) more of the elements in stems on substrate B compared to substrate A without an effect from the substrate on Ca, Fe, LREE and HREEs in this plant tissue. Consequently, element contents in shoots that integrate results from both leaves and stems, respectively, were also affected by substrate showing higher contents of P (10%), Ca (18%), Mn (170%), Fe (23%) and LREEs (60%) and HREEs (13%) in shoots of plants that were cultivated on substrate B compared to plants on substrate A.

The element contents in shoot biomass were not influenced by general effects of culture form and P fertilizer addition but rather depended on complex responses of different levels of plant tissue accumulation based on interactions of culture form and substrate properties as well as additional interaction effects of P fertilizer amendment (Table 7). Specifically, compared to *L. angustifolius*, intercropping with *L. albus* did not positively affect the accumulation of the investigated elements except that of Mn in leaves and shoots of barley plants on substrate B. On substrate B, the presence of *L. albus* increased Mn content in leaves by 116% and in shoots by 63% compared to monocultures, while on substrate A, *L. albus* increased the leaf Mn contents by 102% compared to monocultures. However, for LREEs and HREEs, *L. albus* significantly decreased the element contents in shoots (by 68% and 71%, respectively) and leaves (by 36% and 46%, respectively) when the plants grew on substrate B with 3 g m^{-2} P addition, while on substrate A, no effect of *L. albus* on REE accumulation in mixed cultured barley was observed.

Unfortunately, in this study, *L. albus* was solely cultivated on the two substrates with higher dosing of P fertilizer and, thus, further evaluations of responses of the mixed cultures to different P availabilities are not possible. However, considering mixed cultures with *L. angustifolius*, the effect of intercropping on element accumulation was strongly dependent on the growth substrate and P fertilizer addition. More specifically, on both substrates, there was no response of mixed cultured barley regarding the contents of P, Ca, Mn and Fe when barley and *L. angustifolius* were cultivated

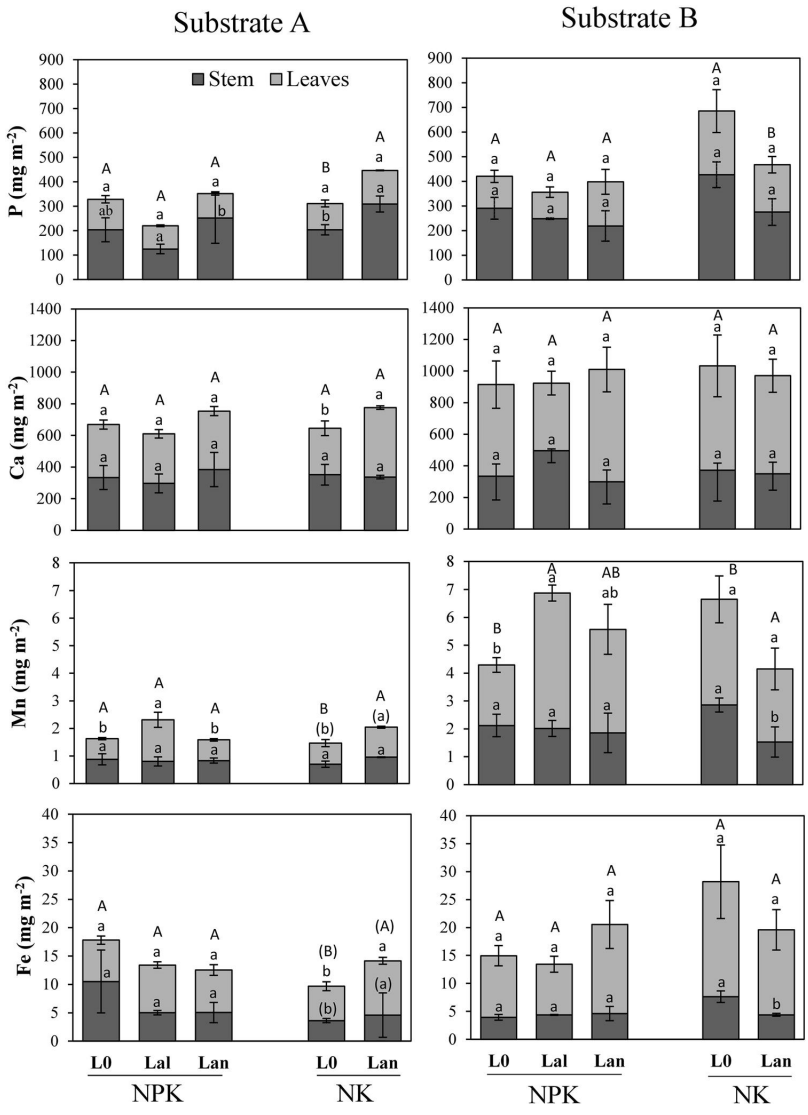

Fig. 1 Total accumulation of nutrients in leaves, stems and shoots (total height of bars) of barley plants in monocultures (L0) and mixed cultures with *L. angustifolius* (Lan) or *L. albus* (Lal) on slightly alkaline substrate A and slightly acidic substrate B. On both substrates, the plants in different culture forms were treated with 3 g m^{-2} P (NPK) or 1.5 g m^{-2} P (NK). Means ± SD ($n = 5$). Differences among means were identified by MANOVA followed by Duncan's post hoc test. Small letters denote differences in element contents within a specific plant part, substrate and P addition treatment. Capital letters show differences between shoot contents within the substrates and treatments at $\alpha = 5\%$

with a higher supply of P (NPK treatment). In contrast, when P supply was reduced (NK treatment) and barley was cultivated neighbouring to *L. angustifolius*, shoot contents of P, Mn and Fe increased on substrate A by 64% (P), 56% (Mn) and 62% (Fe). This was mostly caused by a significant increase in leaf contents, except for P, whereas on substrate B, the shoot contents of P, Mn and Fe decreased by 37% (P), 50% (Mn) and 37% (Fe), respectively, due to decreased accumulation in stems and leaves. Concomitantly, on substrate B, there were clear tendencies of a reduction of shoot LREE (by 44%) and HREE (by 46%) accumulation when plants were cultivated with *L. angustifolius* and 1.5 g m^{-2} P dosing compared to the monocultures. Under these conditions, *L. angustifolius* significantly reduced LREE contents in stems of barley by 69%. Also, on substrate B, the presence of *L. angustifolius* significantly reduced stem contents of HREEs by 46% in 3 g P m^{-2}–dosed mixed cultures compared to the monocultures but without striking effects on bulk shoot contents which remained unchanged.

In contrast, on substrate A, mixed cultures with *L. angustifolius* significantly increased contents of LREEs (by 79%) and HREEs (by 96%) in shoots of barley

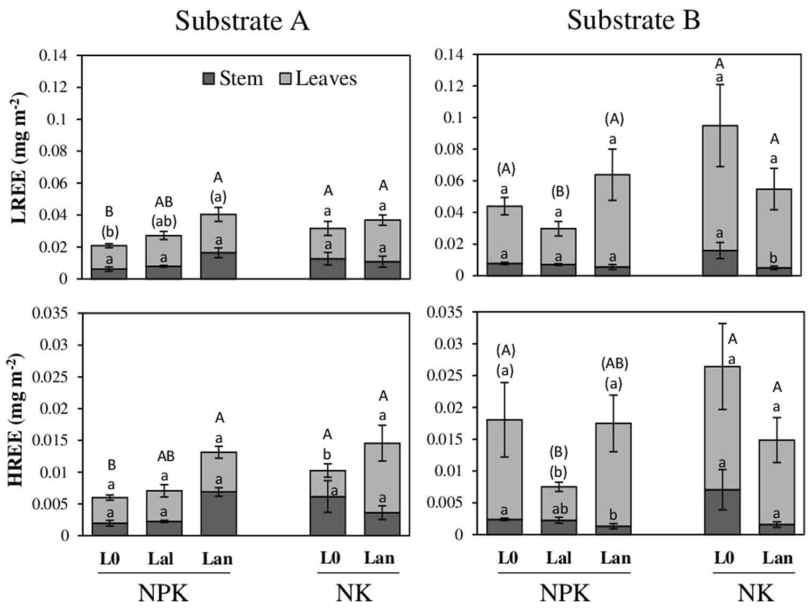

Fig. 2 Total accumulation of nutrients in leaves, stems and shoots (total height of bars) of barley plants in monocultures (L0) and mixed cultures with *L. angustifolius* (Lan) or *L. albus* (Lal) on slightly alkaline substrate A and slightly acidic substrate B. On both substrates, the plants in different culture forms were treated with 3 g m^{-2} P (NPK) or 1.5 g m^{-2} P (NK). Means ± SD ($n = 5$). Differences among means were identified by MANOVA followed by Duncan's post hoc test. Small letters denote differences in element constants within a specific plant part, substrate and P addition treatment. Capital letters show differences between shoot contents within the substrates and treatments at $\alpha = 5\%$

Table 7 Multifactor multivariate ANOVA based on leaf and stem contents (µg m^{-2}) of barley plants, exploring for effects of the growth substrate, fertilizer addition (3 g m^{-2} P or 1.5 g m^{-2} P, respectively) and culture form (monocultures and mixed cultures)

Plant tissue	Source of variation	P	Ca	Mn	Fe	LREE	HREE
Leaves	Substrate	*	**	***	**	**	**
	Fertilizer	NS	NS	NS	NS	NS	NS
	Culture	NS	0.08	**	NS	(*)	*
	Substrate × culture	NS	NS	NS	NS	NS	(*)
	Fertilizer × culture	NS	NS	NS	NS	NS	NS
	Substrate × fertilizer × culture	(*)	NS	*	(*)	NS	NS
Stems	Substrate	(*)	NS	***	NS	NS	(*)
	Fertilizer	NS	NS	NS	NS	NS	NS
	Culture	NS	NS	NS	NS	NS	NS
	Substrate × culture	**	NS	(*)	NS	*	*
	Fertilizer × culture	NS	NS	NS	NS	(*)	NS
	Substrate × fertilizer × culture	NS	NS	NS	(*)	NS	NS
Shoots	Substrate	(*)	(*)	***	(*)	*	(*)
	Fertilizer	NS	NS	NS	NS	NS	NS
	Culture	NS	NS	NS	NS	NS	NS
	Substrate × culture	**	*	**	(*)	**	**
	Fertilizer × culture	NS	NS	NS	NS	NS	NS
	Substrate × fertilizer × culture	*	NS	*	*	NS	NS

NS not significant

(*)$p < 0.1$; *$p < 0.05$; **$p < 0.01$

compared to the monocultures. This can be attributed to a combination of increasing contents in leaves (60% increase for LREEs and 50% increase for HREEs) and in stems (169% increase for LREEs and 263% increase for HREEs) when 3 g m^{-2} P was given. For HREEs, this effect was also visible in leaves of plants that were treated with lower P doses (62% increase). However, the effect in leaves was not strong enough to influence bulk shoot contents of HREEs that remained unchanged compared to the monocultures. Due to a decrease in stem HREE contents, there was no effect on LREE plant stands treated with 1.5 g m^{-2} P.

Phosphorus concentrations in lupin plants as affected by substrate and P supply

Mixed cultures of barley and lupins that received only low doses of P (1.5 g P m^{-2}) did not show significant differences in leaf P concentrations when plants cultivated on substrates A and B were compared (Fig. 3). Nevertheless, P concentrations in plants on substrate B were slightly higher (2.3 mg g^{-1}) compared to lupins cultivated on substrate A (1.9 mg g^{-1}). Generally, on both substrates, fertilization of the mixed cultures with P fertilizer significantly increased the concentrations of P and this effect was most visible on substrate B where NPK-treated plants reached up to 3.1 mg g^{-1} P in leaves. Here, plants of *L. angustifolius* displayed substantially higher P concentrations than plants on substrate A. *L. albus* was only cultivated under the NPK addition of substrate A, and thus, investigations of responses of the species to substrate and P supply were not possible. Compared to *L. angustifolius*, *L. albus* exhibited similar P concentrations when both species received NPK fertilizer (Fig. 3).

Discussion

Evaluation of carboxylate release in different lupin species

In the greenhouse experiment, exudation experiment was carried out as a means to evaluate the carboxylate release and, consequently, the nutrient acquisition efficiency of the cultivars of *L. albus* (Feodora) and *L. angustifolius* (Sonate)

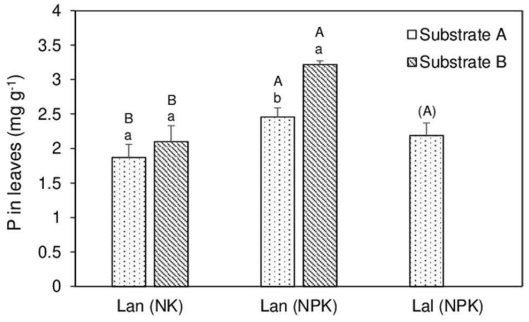

Fig. 3 Leaf P concentrations in mixed cultured lupin plants (*L. angustifolius* (Lan), *L. albus* (Lal)) that received fertilizer with 1.5 g P m^{-2} (NK) or 3 g P m^{-2} (NPK), respectively. Means ± SD ($n = 4$). Significant differences among means were identified by *t* tests with Bonferroni adjustment. Small letters denote differences between the substrates within a certain P treatment. Capital letters show differences in P treatments within a specific substrate. Means with different letters are significantly different at $\alpha = 5\%$

that were later used in the field experiment for intercropping with barley. Lupins are characterized by an extraordinarily high efficiency to mobilize sparingly available P, Fe and Mn in the rhizosphere through carboxylate release and acidification which is extensively documented in the literature (Cu et al. 2005; Lambers et al. 2013; Pearse et al. 2006; Wiche et al. 2016b), while barley is described as P-inefficient (Marschner 1995). The results successfully demonstrate that the response of the two species was divergent, showing a higher release of carboxylates in *L. albus* under P-deficient conditions, whereas *L. angustifolius* responded with a decreased release of carboxylates and the highest exudation rates under high P supply (Table 3). For *L. albus*, this is in congruency with the results from Pearse et al. (2006), Müller et al. (2015) and Neumann and Römheld (2000) who reported increased diffusion of citrate and malate as a consequence of metabolic shifts in carbohydrate allocation from shoot to roots in concert with increased biosynthesis of malate and citrate and decreased citrate turnover in the tricarboxylic acid cycle. Concomitantly, the decreased release of carboxylates in *L. angustifolius* suggests that this species (or the selected cultivar) lacks the ability to alter carboxylate metabolism following P deficiency similar to chickpea and *Brassica napus* (Pearse et al. 2006; Lambers 2022). Indeed, the total amounts of carboxylates released per plant were higher in *L. albus* whereas the exudation rates (per root dry weight) of both lupin species were similar under low P supply (Table 3). *Lupinus albus* is a cluster root-forming lupin species and generally produces more extensive root systems compared to *L. angustifolius* (Egle et al. 2003; Pearse et al. 2006; Clements et al. 1993). Since carboxylate release mainly concentrates on active cluster roots, the lower carboxylate release per unit root weight in *L. albus* observed in this study could be explained by a higher total root dry mass relative to the number of active root tip regions of *L. albus* in concert with no changes in biomass allocation under P-deficient conditions (Funayama-Noguchi et al. 2015). This partly contradicts the previous findings of Pearse et al. (2006) who observed higher rates of carboxylate release per unit root mass in *L. albus* compared to *L. angustifolius*. We emphasize that the ability to respond to P deficiency varies substantially among different lupin species and even different genotypes within a species. More specifically, Egle et al. (2003) explored P supply–induced changes in malate and citrate release of different cultivars of *L. albus* and *L. angustifolius* and demonstrated a higher variation for *L. angustifolius* than for *L. albus*. The latter was characterized by a lower carboxylate release efficiency per unit root and responded to P deficiency with elevated carboxylate release, while all *L. angustifolius* cultivars showed the opposite response (Egle et al. 2003), which is in good agreement to our results. Here, adequately P-supplied *L. angustifolius* showed substantially higher carboxylate exudation rates and amounts

of citrate released per plant individual compared to *L. albus* (Table 3). However, cultivar-dependent differences between our study and that of Pearse et al. (2006) cannot a priori be ruled out. Based on the above, it appears that *L. albus* should be preferably selected for intercropping aiming at improved plant nutrition in mixed culture systems, especially when plant growth is limited by P availability. On the other hand, the tested *L. angustifolius* cultivar seems to be suitable for improvement of nutrient supply on moderately fertile soils. With regard to a selection of lupin species, other substrate parameters, particularly soil pH, Ca and bicarbonate concentrations, are of additional relevance. Compared to *L. angustifolius*, *L. albus* is relatively tolerant against Ca and bicarbonate in soil solution and develops well on soils over a wide pH range from 5 to 8. However, in alkaline soils above a pH value of 7, iron deficiency can cause chlorosis (Duthion 1992). In contrast, *L. angustifolius* is calcifuge and high concentrations of bicarbonate may decrease root growth and increase carboxylate release, irrespective of the external P supply (Peiter et al. 2000).

Effect of substrate properties on plant growth and nutrient availability to the plants

Considering the leaf nutrient concentrations which are commonly used as proxies for the nutritional state of plants (Hayes et al. 2014), it was obvious that on both substrates, the barley plants suffered from Mn and P deficiency indicated by leaf P concentrations close or even below to the critical value of 2 mg g^{-1} P and 50 µg g^{-1} Mn (Marschner 1995). The lowest concentrations of P and Mn (below 1.9 mg g^{-1} P and 20 µg g^{-1} Mn) were observed in plants on substrate A treated with 1 g P m^{-2} (Table 5). Surprisingly, comparing leaf, stem and shoot biomass on both substrates, we did not observe significant changes in plant yields between the substrates (Tables 4 and 5). Compared to substrate A, concentrations of P, Ca, Mn and Fe in barley leaves as well as bulk shoot contents (Fig. 1, Table 5) were significantly higher on substrate B, indicating an improved nutrient supply on this substrate with its slightly acidic pH. Furthermore, on substrate B, leaf P concentration of lupin plants was significantly higher than that on substrate A and significantly higher compared to *H. vulgare* (Table 5, Fig. 3), while on substrate A, leaf P concentration in unfertilized plants of *L. angustifolius* was similar to that of *H. vulgare*. Higher nutrient concentrations in lupins compared to *H. vulgare* can be explained by a higher nutrient acquisition efficiency of lupins (Pearse et al. 2006). Based on P concentrations determined by CAL extracts, both substrates were sufficiently supplied with P (Marschner 1995) but the phosphorus was most likely not present in plant-available forms. Substrate A was slightly alkaline (pH 7.9) (Table 1) which fosters the precipitation of sparingly soluble Ca phosphates (Mengel et al. 2001) and low solubility of Mn and Fe. In contrast, soil B was slightly acidic (pH 6.8) (Table 1) so that low specific sorption of P (Mengel et al. 2001) as well as higher solubility of Mn and Fe can be expected (Gupta and Chipman 1976). Generally, higher accumulation and concentrations of the nutrients on substrate B was not surprising (Fig. 1, Table 7). However, the higher availability of the elements on substrate B exhibited by higher tissue concentrations and shoot contents was not a priori predictable based on data of the sequential extraction where substrate A showed lower concentrations of P, Ca, Mn and Fe in mobile, exchangeable fractions (Table 2). On the contrary, substrate B was characterized by higher concentrations of P, Fe and Mn bound into organic matter and amorphous Fe oxyhydroxides (Table 2). This demonstrates that sequential extractions do not sufficiently describe the availability of elements since they do not integrate all soil-associated factors and plant-associated factors overlapping in the rhizosphere in time, space and function (Hinsinger et al. 2009; Vetterlein et al. 2020). This suggests that in this experiment, the higher availability of nutrients on substrate B rather depended on the mobility of the elements in the soil (once they are mobilized) as a consequence of pH and, thus, a lower reprecipitation/readsorption of mobilized elements in the rhizosphere of the plants than distribution of elements in operationally defined element fractions. In this light, we emphasize that CAL extracts (Table 1) exhibited a higher P availability on substrate B which was in agreement with the substrate-induced differences in tissue P concentrations in plants. This suggests that both the CAL–extractant solutions (acidified Ca lactate) and the plants were able to access moderately stable element pools through acidification and ligand–exchange reactions, especially the lupins with their efficient acquisition strategy.

Relationships between the substrate, P fertilization and lupins on plant growth and nutrient availability in mixed cultures

In this experiment, we used a replacement model, where within the mixed cultures, barley was replaced with 11% of *L. albus* and *L. angustifolius* (Wiche et al. 2016a). Although there were slight reductions in yields following a replacement, growth substrate, different levels of P supply and intercropping did not affect plant yields of barley. With the exception of substrate A and on plots with 1.5 g P m^{-2} amendment, intercropping with *L. angustifolius* slightly increased the leaf biomass of barley (Table 5). Of course, plant growth and yield predominantly depend on the nutritional state of the plants which was experimentally controlled by substrate properties, the addition of P fertilizer and intercropping with P-efficient lupins (Lambers 2021). Moreover, the efficiency of intercropping strongly depends

on the nutritional status of both the barley plants and the lupin plants because under conditions of increasing nutrient availability, the barley plants would cover their nutrient demands from soil resources and belowground traits of intercropping plants may not deliver additional benefits. Thus, positive effects of intercropping can be especially expected under conditions of moderate to low nutrient availability. However, as nutrient availability decreases, the root competition intensity between neighbouring plants increases (Schenk 2006; Craine and Dybzinski 2013). Especially in barley–lupin associations, the competing plant individuals are substantially different in morphological and functional traits above and below ground. As such, the resulting competition should be largely asymmetric with the lupins monopolizing soil P and micronutrient sources by exploiting the resource before the barley individuals are able to obtain it (Pearse et al. 2006; Schenk 2006). Consequently, nutrient facilitation in lupin–barley mixed cultures should especially occur in situations where barley is exposed to growth-limiting soil conditions. But, this should be where the lupins are still readily able to satisfy their own nutritional demands (Cu et al. 2005; Gunes and Inal 2009; Wiche et al. 2016b), or when other environmental stress factors and positive effects of barley for the lupins shift the balance between positive and negative interactions (Brooker et al. 2008). Unfortunately, we did not consider other soil resources and environmental factors in our study, and thus, based on our data, no further mechanistic interpretations are possible. In our experiment, the addition of the P fertilizer did not influence the P concentrations and contents of barley plants neither on substrate A nor on substrate B (Tables 4 and 5). Possibly, the differences in doses between the two treatments were not high enough (1.5 g m^{-2} or 3 g m^{-2} P) to generate a treatment-dependent difference in the plants' nutrient supply. Furthermore, the barley plants did not export the P absorbed from roots to shoots (Schjørring and Jensén 1987). Increased P allocation to the grains (El Mazlouzi et al. 2020) influenced the P concentrations in vegetative plant organs, the leaves and stems, respectively. After 8 weeks of plant growth, barley already reached the reproductive stage. Also, based on the above, it is reasonable that the lupin plants strongly competed with barley for phosphate. In fact, the P concentrations in lupins significantly increased when P was added (Fig. 3), indicating a strong root competition for essential elements between lupins and barley. There is evidence that the importance of root competition increases relative to other factors with increasing resource availability in soil (Schenk 2006). Finally, resource facilitation in mixed cultures strongly depends on the nutrient status of the lupin plants, their responses through the release of carboxylates influencing the solubility of the elements in the rhizosphere and migration of elements between the intermingling root systems (Cu et al. 2005; Wiche et al. 2016a, 2017a). The availability of P and micronutrients was higher in substrate B than in substrate A (Table 1, Fig. 1). Therefore, the low performance of *L. angustifolius* and *L. albus* in mixed cultures with barley on substrate B (Fig. 1, Table 5) could be explained by the synergetic effects of reduced carboxylate release by the lupins, especially of *L. albus* (Table 3), and higher substrate-induced solubility of the elements fostering element uptake by the barley plants. Nevertheless, increased Mn concentrations and accumulation (Fig. 1, Table 5) in mixed cultured on substrate B indicate that cluster roots of *L. albus* were still active even when P fertilizer was added. It has to be noticed that even on substrate B, the plants were still undersupplied with Mn (Table 5, Section "Effect of substrate properties on plant growth and nutrient availability to the plants") which is an additional factor triggering carboxylate release by lupins (Marschner and Römheld 1994; Lambers et al. 2013, 2015). Concomitantly, carboxylates of *L. albus* are known to strongly affect the availability of Mn as this species is considered a hyperaccumulator of Mn (Lambers et al. 2015). In this regard, lacking effects in mixed cultures with *L. angustifolius* might indicate a lower ability of *L. angustifolius* to respond to deficiency of Mn, while decreased accumulation of P and Mn in the presence of *L. angustifolius* could be due to the competition of barley and lupins for these nutrients.

On substrate A, intercropping with *L. angustifolius* slightly increased leaf P concentrations of low P–dosed plants above the critical level of 2 mg g^{-1}, suggesting that the improved nutritional state of the barley plants was responsible for the increase in leaf biomass (Table 5). On this alkaline substrate, leaf and shoot nutrient concentrations and contents of barley were exclusively positively affected (Table 5, Fig. 1) on experimental plots with 1.5 g m^{-2} P addition although the leaf P concentrations of lupins suggested a lower P supply in *L. angustifolius* (Fig. 3) which should lead to decreased root activity of this lupin species (Table 3). However, in plots with a higher P supply, we observed a better plant growth of lupins (data not shown here) so that it is reasonable that the mobilized nutrients were initially taken up by the lupins without any positive effects on barley. Concomitantly, increased concentrations and accumulation of Ca, Mn and Fe in mixed cultures with lower P supply (Table 5, Fig. 1) most likely originated from resource facilitation under the growth-limiting conditions of substrate A, where neighbouring lupins improved the nutritional status of barley plants.

Effect of substrates, P fertilization and lupins on the availability of REEs in mixed cultures

In soils, REEs share many chemical similarities with essential plant nutrients, especially calcium (Brioschi et al. 2013; Censi et al. 2014, 2017; Martinez et al. 2018; Wyttenbach

et al. 1998). Thus, nutrient-bearing soil phases such as phosphates, organic matter and Fe oxyhydroxides are important hosts for these elements (Diatloff et al. 1993; Zhimang et al. 2000; Cao et al. 2001; Wiche and Heilmeier 2016; Wiche et al. 2016b). Accordingly, in the soil used for the field experiment, REEs were mostly present in fractions 3–5 and with slight enrichment in fraction 3 of substrate B (Table 2). Low soil pH and the presence of dissolved organic matter strongly impact the mobility and plant availability of REEs (Diatloff et al. 1993; Zhimang et al. 2000; Cao et al. 2001; Tyler and Olsson 2001; Pourret et al. 2007; Kovaříková et al. 2019). As such, the higher concentrations (Table 6) and accumulation (Fig. 2) of REEs on substrate B in comparison to substrate A can be attributed to a higher solubility of the elements in this soil. Higher accumulation of LREEs relative to HREEs observed in this study (Table 6, Fig. 2) closely follows the natural abundance of the elements in the substrates (Table 3). Furthermore, the literature indicates a preferential uptake of LREEs compared to HREEs (Censi et al. 2017; Martinez et al. 2018) due to the higher stability of HREE–organic complexes and stronger adsorption of HREEs at ion exchange sites in the soil. These, in turn, may have contributed to these results. Surprisingly, in this study, leaf concentrations of REEs were constantly higher than stem concentrations and the plants mostly responded by changes in leaf REE concentrations (Table 6). Although the literature indicates a clear trend of decreasing REE concentrations in the order roots > stems > leaves across many plant species and genera (Li et al. 2001; Wen et al. 2001; Xu et al. 2003; Tyler 2004; Brioschi et al. 2013; Yuan et al. 2018), some studies also reported a reversed concentration pattern showing higher concentrations in leaves than in stems, especially in cereals such as oat, wheat and rice (Wiche et al. 2016a, b; Kovaříková et al. 2019). Thus, different REE patterns among different plant species may reflect a species-specific mobility of REE within plants (Kovaříková et al. 2019) and our findings in barley support the described pattern for cereals.

Differences in substrates as well as intercropping with lupins impacted both leaf and bulk shoot contents of barley (Fig. 2), although in barley, the predominant portion of the shoot biomass consisted of stems (Table 5). Leaves only accounted for one-third of the total shoot biomass (Table 5), and changes in foliar REE absorption due to treatment measures were impactful enough to compensate the lower biomass of this plant part when total shoot contents are considered (Fig. 2). Similar to the findings for nutrients (see Section "Effect of substrate properties on plant growth and nutrient availability to the plants"), REE concentrations on substrate B were predominantly influenced by substrate without significant effects of P fertilizer addition or positive effects of lupins in mixed cultures. However, on substrate B, the presence of *L. albus* significantly decreased both shoot REE concentrations and contents, especially when the plants were fertilized with P which highlights an immobilization or uptake of the elements by the lupins under conditions where mobility of the elements is high. Unfortunately, our experimental design did not allow exploring the processes beyond these effects. Nevertheless, it is reasonable that the lupines with their extensive root systems and especially *L. albus* which produces more extensive root systems compared to *L. angustifolius* (Clements et al. 1993) did not only compete for essential elements such as P but also REEs. Although lupines are generally characterized by low shoot REE absorption so far (Wiche and Heilmeier 2016), their roots could represent important element sinks in soil where REEs are stored or adsorbed onto cell structures (Han et al. 2005), especially when root carboxylate release diminishes due to sufficient external P supply (Table 3).

On alkaline substrate A, the addition of P fertilizer significantly reduced both LREE and HREE concentrations in monocultured barley plants (Table 6). This can be attributed to a precipitation of the elements as hardly soluble REE phosphates at alkaline conditions (Saatz et al. 2016; Han 2020) or a "dilution" effect originating from slightly higher shoot biomass (Table 5) which is frequently reported for non-essential elements (Chien and Menon 1995). Compared to the monocultures, the presence of *L. angustifolius* significantly increased tissue concentrations and shoot contents of both LREEs and HREEs in mixed cultured barley. Increased REE availability in mixed cultures with lupins was already described by Wiche et al. (2016b) but without considering differences in substrates or nutrient availability. In the present study, positive effects of mixed cultures were only visible on the alkaline, P fertilizer–amended soil and in the presence of *L. angustifolius* which releases higher amounts of carboxylates under sufficient P supply (Table 3). Indeed, in view of the P-induced increase in carboxylate release observed in the greenhouse study (Table 3), these results were consistent with our previous findings (Wiche et al. 2016a, b); however, compared to *L. albus*, *L. angustifolius* is much less tolerant against high bicarbonate concentrations present at high soil pH as it can be expected in soil A (Peiter et al. 2000). High concentrations of bicarbonate can reduce the formation of lateral roots in *L. angustifolius* and may increase the carboxylate release in this calcifuge lupin species as it has been reported for lime-intolerant *Lupinus luteus*. However, Peiter et al. (2000) and Egle et al. (2003) reported a large variation in root responses among different *L. angustifolius* cultivars. In contrast, *L. albus* generally tolerates relatively high soil lime contents and does not respond to liming with reduced root growth and elevated carboxylate release (Peiter et al. 2000). Thus, the missing effects of *L. albus* on REE accumulation by barley can be widely explained by a reduced carboxylate exudation of *L. albus* due to sufficient P supply, while it seems reasonable that the

significant effects of *L. angustifolius* are a consequence of carboxylates and protons released into the soil affected by high P supply and/or the bicarbonate in alkaline soil A. Most probably, under these conditions, the carboxylates released by lupins mobilized the REEs through the formation of soluble REE–carboxylate complexes (Wiche et al. 2017a) in the rhizosphere of the lupins. Since REEs are not essential for plant growth (Tyler 2004) and complexes of REEs are discriminated relative to their ionic forms during plant uptake (Han et al. 2005; Wiche et al. 2017a), the complexes were obviously not adsorbed by the lupins itself, enabling the movement to the intermingling barley roots where different chemical properties and microbial activity (Neumann and Römheld 2000; Renella et al. 2004) might have fostered the decay of complexes and thus root uptake and transport of REEs to the shoots of intercropped barley.

Conclusion

We could demonstrate that soil-associated factors above plant-associated factors play a crucial role in determining REE fluxes in soil plant systems. Within a certain soil environment, application of 3 g P m^{-2} reduced the accumulation of REEs in barley monocultures, most likely through REE precipitation in the root zone. However, our results clearly show that P availability also indirectly affects REE fluxes in soil–plant systems by influencing the nutritional status of the plants, and thus, the chemical properties of the *meta*-rhizospheres of intermingling barley–lupin root systems. In barley–lupin associations, the mobilization of REEs in the rhizosphere of lupines and REE transport to neighbouring plants seems to depend on the species-specific ability to respond to different levels of P supply with carboxylate release. *L. angustifolius* cv. Sonate, a lupin cultivar that responds to increased P supply with increased carboxylate release, increased the accumulation of REEs in barley plants when the plants were additionally supplied with P fertilizer and cultivated on an alkaline soil characterized by low initial availability of REEs and nutrients. In contrast, on soil with high P and REE mobility, the presence of *L. albus* cv. Feodora, which responded to increased P supply with decreasing carboxylate release led to decreased REE contents in barley, most probably due to the root REE absorption of the lupins. Considering these factors, mixed culture cropping systems could be a powerful tool to enhance the accumulation of REEs in a sense of phytoremediation or phytomining on marginal soils, while at the same time, the mixed cultures with *L. albus* cv. Feodora could be deployed to attenuate REE accumulation in crop plants for food production, especially in REE-polluted soils. The processes involved in the results are not yet fully understood, and thus, elucidation of chemical element species in the rhizosphere of neighbouring plants and responses of different cultivars to P supply–induced REE mobilization remains a field of further research. Nevertheless, our findings suggest that interspecific root interactions involved in REE fluxes in legume–grass communities are influenced by species-specific strategies related to P acquisition and the nutritional status of neighbouring plant individuals.

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Data availability Not applicable. Raw data (primary data obtained from HPLC or ICP-MS) has not been considered for publication in data repositories.

Declarations

Ethics approval and consent to participate The authors declare that this work is original, has not been published previously and is not under consideration for publication elsewhere. The authors declare that this work fulfils the good scientific practice according to the Committee on Publication Ethics (COPE). All authors made substantial contributions to the conception or design of the work and to the analysis and interpretation of the data. All authors drafted the work or revised it critically for important intellectual content.

Consent for publication All authors approved the version to be published and agreed with the content, gave explicit consent to submit and obtained consent from the responsible authorities at the institute/organization where the work has been carried out. All authors agreed to be accountable for all aspects of the work.

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Selective removal of selenium by phytoremediation from post/mining coal wastes: practicality and implications

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ABSTRACT

Selenium (Se), a metalloid typically natural in origin, is also present in coal washery by-products such as fly-ash stockpiles. The removal of Se in coal washery by-products can be achieved through various bio-physico-chemical processes. This study investigated the phytoremediation of Se from post coal process wastes using *Brassica juncea* species. The selected plant species were grown in coal process wastes enriched with either a growth soil mix or hydroponic substrates. Successful Se extraction (48% and 28%) was achieved from both mixes. The tested plant species also accumulated other heavy metals (Arsenic, Cadmium, Copper, Lead) along with selenium in the plant biomass.

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Phytoremediation; phytoextraction; selenium; *Brassica juncea*; coal wastes

1. Introduction

Selenium (Se) is described as a metalloid commonly found in sedimentary rock formations; mostly in arid regions with significant concentrations [1]. This is one of the trace elements found in coal washery by-products, has become an element of focus, especially in soil treatment [2]. It has been identified as one of the main contributors to the decline in the profitability of coal washing processes [3]. Selenium can either be natural or anthropogenic in origin [4]. The increase of Se concentration in the soil is usually triggered by anthropogenic processes such as coal burning or treatment [5]. Bodnar et al. (2012) described the necessity of Se in living organisms, and these benefits are highlighted with the discrepancies that are brought by the deficiency or excessiveness of selenium [6]. Though beneficial for living organisms, the range between safe and toxic dosage is narrow, where concentrations as high as 4–5 mg/kg of Se can be toxic to plants and living organisms. On the contrary, in humans, Se deficiencies have been calculated to occur when dietary intake is <0.04 mg Se/day [7].

There are various biological, chemical and physical treatment technologies, which could be applied to achieve effective Se removal. However, these results in secondary contaminants, which need further treatment, and are limited to the treatment of low Se concentrated solutions [8]. The most commonly used methods include soil washing [9], excavation, and storage of the contaminated sediments [10]. Phytoremediation provides an efficient alternative treatment option to physical, chemical and other biological treatment technologies [11,12]. Phytoremediation has been proved to be cost-effective, environmentally and aesthetically feasible. This method also has the ability to significantly limit the amount of contaminant released to the environment [11,13]. Furthermore, the harvested Se rich plant biomass could be used as a secondary supply of nutritious Se for animals in Se deficient regions [14].

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Phytoremediation can be achieved through *rhizofiltration, phytostabilisation, phytovolatisation* and *phytoextraction* [15] and the definition of each is well described in past studies [15–19]. Among the aforementioned, *phytoextraction* is widely used as a process to achieve the removal of inorganic contaminants such as cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), zinc (Zn), cobalt (Co), nickel (Ni), Se and arsenic (As) from the soil [16]. In this process the contaminant gets absorbed from the soil through the roots, from which it is transferred and then accumulated in the shoots. This process requires the constant harvesting of the plants to elude the spread of contaminants [20]. Plant selection is important in phytoremediation, as the selected plant should be able to carry out the specified phytoremediation mechanism to remove the contaminants. Different plant species vary intensively in terms of the ability to retain and tolerate metals and hyperaccumulation [21]. They grow rapidly with a high biomass yield, and are usually available as per habitat preference. A hyperaccumulating plant should have the ability to distinguish the targeted metal from the others with similar chemical properties (e.g. being able to distinguish Se from sulphur compounds) [22]. The main reason for this is to ensure that no secondary contamination from biofortification either in soil supplementation or Se-enriched plant biomass [23].

The plant species, *Brassica juncea (B. juncea)*, also known as Indian mustard is an oil crop in the *Brassicacea* family [24] which has been used for the phytoremediation of heavy metals such as Cd, Cr, Pb and Se [24,25]. In the aforementioned studies, the plant showed great resistance against heavy metals. Biofortification is a process in which fertilisers, genetically modified plants, and other plant genetics are used to achieve effective uptake as well as accumulation of nutrients in plants. The combination of biofortification and phytoremediation achieved a higher Se uptake and thus used the harvested plant biomass for other purposes such as producing fertilisers [21]. The past studies also highlight that *B. juncea* has the ability to accumulate a minimum of 13% to 48% Se [20].

The present study is conducted with an objective of determining the ability of *B. juncea* to extract bioavailable Se from soil contaminated with coal process wastes through phytoextraction. The study investigated the feasibility of the selected plant species to accumulate Se without capturing other heavy metals. The collective goal of this work is to consolidate data that could be used as a groundwork reference for further studies in phytoremediation of soil contaminated with coal wastes.

2. Materials and methods

2.1. Materials

2.1.1. Soil samples

The samples used are from the dried tailings pond of the Bowen Basin coal mine, located in Queensland, Australia. Three samples were collected randomly from different parts of the tailing pond. A seed raising mix (Debco Seed Raising Mix from Flower Power Garden Centres Pty Ltd) was used to dilute the toxicity of the coal samples. For hydroponic tests, coco coir potting mix (Debco Coir Peat also from Flower Power Garden Centres Pty Ltd.) a mixture of coconut skins and perlite was used.

2.1.2. Experimental equipment

The set-up consists of a growth chamber (200 x 70 × 150 cm) built with steel frames that were lined inside with roof sarking to allow sufficient lighting. The main purpose of using the growth chamber was to control the temperature and light within the system. Four white UV lights were installed (1 m above the ground with 60 cm gaps placed on each end) to get even distribution of light within the system. Two rows of flower pots were aligned directly underneath the UV lights. The lights were left ON for 18 h/d in order to keep the growth chamber warmer or at a consistent temperature for the full day, even when the lights were turned OFF.

2.2. Methods

2.2.1. Preliminary germination tests

Coal ash samples lack a structure, have no micronutrients, and inconsistent pH values; thus, all these properties could affect the plant growth [26]. As this is the case, preliminary germination tests were carried out to find the correct ratios at which the coal soil should be amended with soil for effective seed germination. The germination tests were carried in three ratios (25%, 50% and 75%) and a 50:50 soil to sample ratio was found to be the most suitable.

The chemical compositions of the coal process waste samples were determined by ICP-MS analysis, using microwave digestion (PerkinElmer Nexion) (Table1).

2.2.2. Planting

There were control tests, prepared both for the hydroponic and soil mix (seed growth mix). The coal process waste samples were amended with a seed growth mix for the normal tests, and 7 g of the *B. juncea* (Indian mustard seeds). Each pot was watered daily to ensure that the soil remained moist to support seed germination, including the control tests to maintain consistency. The soil nutrients were prepared and spread all over the samples. Similarly, a sterile/non-reactive hydroponic nutrient was prepared to feed all the coco coir samples. The soil-based tests were watered on three times in a week and fed by the nutrients at the end of every second week as per the manufacturer's instructions. The hydroponic tests were watered on similar days and fed with their respective nutrients.

2.2.3. Sample collection and preparation for chemical analysis

Harvesting was carried out after 10 weeks when the plants had fully matured. The first two-three weeks are allocated to seed germination and the rest are for the entire growth period. In most cases, at the mentioned period the plants have fully matured [20]. The plants were harvested from the tips at the immediate contact with the soil, in order to limit cross-contamination between the plant and the soil. The entire plant was removed from just above the roots. These were rinsed with deionised water and dried. The dehydration and drying process was done in an oven at 60°C for 48 hours. The dried biomass was then subjected to microwave digestion and then sent to the Mark Wainwright Analytical Centre at the University of New South Wales (UNSW) (Australia) for the dissolved metal concentration analysis (ICP-MS). The toxicity characteristic leaching procedure was run at the beginning, halfway and at the end of the experiment to monitor unstable selenium.

2.2.4. Determination of bioconcentration factor and bioaccumulation factor

The bioconcentration factor (BCF) is the ratio used to determine the proficiency of the selected plant species to uptake the targeted metalloid (Se) and the mathematical definition to determine the BCF is below [27]:

$$BCF = \frac{Metal\ concentration\ in\ the\ entire\ plant\ tissue}{Initial\ metal\ concentration\ in\ soil} \quad (1)$$

Table 1. Major elemental composition ($mg.kg^{-1}$) of the coal tailing pond samples for phytoremediation.

	T1[a] ($mg.kg^{-1}$)	T2[a] ($mg.kg^{-1}$)	T3[a]($mg.kg^{-1}$)
As	33.38	10.46	26.42
Cd	0.69	0.20	0.92
Cu	18.50	12.20	38.86
Pb	51.01	10.56	23.99
Se	7.70	1.78	6.21

[a]Where T1, T2 and T3 are three samples from different parts of tailing pond

Whereas the biological accumulation coefficient (BAC) is the concentration of metalloid (Se) in the plant shoots divided by the metal (Se) concentration in the soil and the mathematical definition to determine the BAC is below [27]:

$$BAC = \frac{Metal\ concentration\ in\ shoots}{Metal\ concentration\ in\ soil} \tag{2}$$

3. Results

After allowing the plants to grow for 10 weeks, samples were collected and subjected to Se concentration analysis from both the plant biomass and the soil of the different tests. For all individual samples collected, a net positive Se uptake in the biomass, and a reduction in the soil's Se concentration were observed. Due to the cultivation and preparation process, the present study could not determine the exact portion of shoot where the majority of Se and/or other metals accumulated. This would be potentially important in the event of large-scale planting, such as a mining area where mowing and regrowth cycles could be incorporated. The initial metal composition of the coal tailing pond samples used for phytoremediation study, measured by ICP-MS is given in Table 1.

3.1. Se accumulation in plant biomass

The Se concentration accumulated within the plant biomass after the harvesting cycle for three different tailing pond samples are shown in Figure 1. For this, the accumulated Se concentrations in both soil and hydroponic plants are determined after harvesting and compared with the initial Se concentration of each tailing. The plants grown in the hydroponic system seem matured faster than in the soil system. At the initial time of planting the concentrations of Se within the samples were: 7.7, 1.78 and 6.21 $mg.kg^{-1}$ in T1, T2 and T3 respectively. The overall uptake of Se with-in the plant biomass was found to be higher in the soil mix tests than the hydroponic. Nevertheless, the quantity of the accumulated Se from each tailing differed due to the change in initial Se concentration from sample to sample, mainly because the samples were collected from different parts of the tailing pond. This may be attributed to the different environmental conditions, such as leaching, additionally the coal washery material may be from different processes.

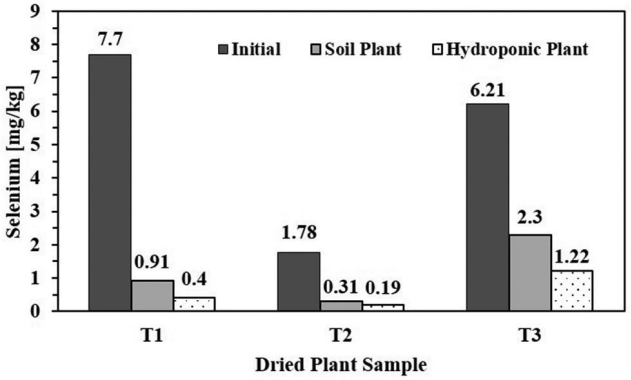

Figure 1. The concentration of the Se accumulated in the plant biomass for both soil and hydroponic tests, where T1, T2 and T3 represent the three samples from the different parts of the tailing pond.

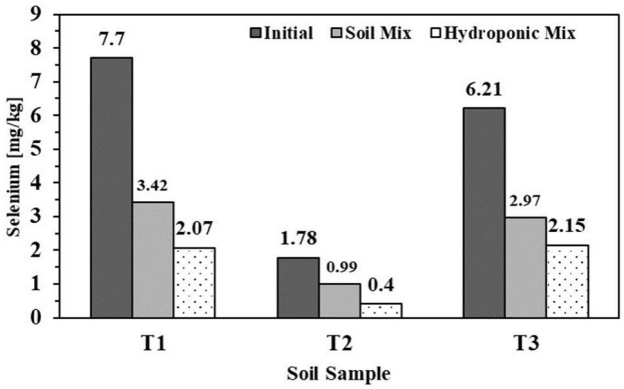

Figure 2. Selenium concentrations found within the soil sample from both the soil mix and hydroponic tests. Where T1, T2 and T3 represent the three samples from the different parts of the tailing pond.

Table 2. The total concentration ($mg.kg^{-1}$) of the As, Cd, Cu, Pb and Se for the different soil measure from the soil mix and hydroponic plant biomass.

Sample label	Soil Plant Biomass				Hydroponic Plant Biomass			
	As	Cd	Cu	Pb	As	Cd	Cu	Pb
T1	0.61	0.33	4.18	0.67	0.78	0.34	6.36	1.44
T2	0.27	0.30	3.90	0.36	0.14	0.64	4.21	0.45
T3	0.92	0.52	6.60	0.83	0.11	0.28	3.91	0.35

3.2. Selenium concentration in the soil samples after harvesting

The Se concentration profile of the soil samples taken from both the soil and hydroponic tests is illustrated in Figure 2. The two used growth environmental conditions (hydroponic and soil mix) showed different behaviours. The results indicate that significant changes of the Se concentration in the soil which was noted from the tested plant biomass. The average Se content remaining within the three test samples are 48% and 28% in the soil mix and hydroponic tests respectively. The change in the heavy metal concentration within the soil cannot be fully accounted by the Se accumulated within the plant biomass. Nevertheless, the Se speciation to determine which type of Se mostly accumulated within the soil and plant biomass is out of the scope of present study. Therefore, the Se reported in this study is total Se as followed from a previous study [28].

3.3. Bioconcentration factor of heavy metals retained

The results showed that there were other toxic heavy metals such as As, Cd, Cu and Pb were also accumulated from the soil to the plant biomass. On an average the highest accumulated metal from the three tailings is Cd in both the normal soil and hydroponic plant biomass. The BCF was calculated from the values in Tables 1 and 2, using the formula (1). The values are illustrated in Figure 3 (a and b) respectively. A high BCF for Cd (3.20) is observed in both the hydroponic test and normal soil tests, followed by Cu (0.34). Nevertheless, the BCF for As is found to be negligible with a value of 0.01.

4. Discussion

It is important to note that the Se accumulated in the plant biomass was a fraction of the initial Se in the soil. Several plants have the ability to accumulate moderate amounts of Se in their shoots [7]. An

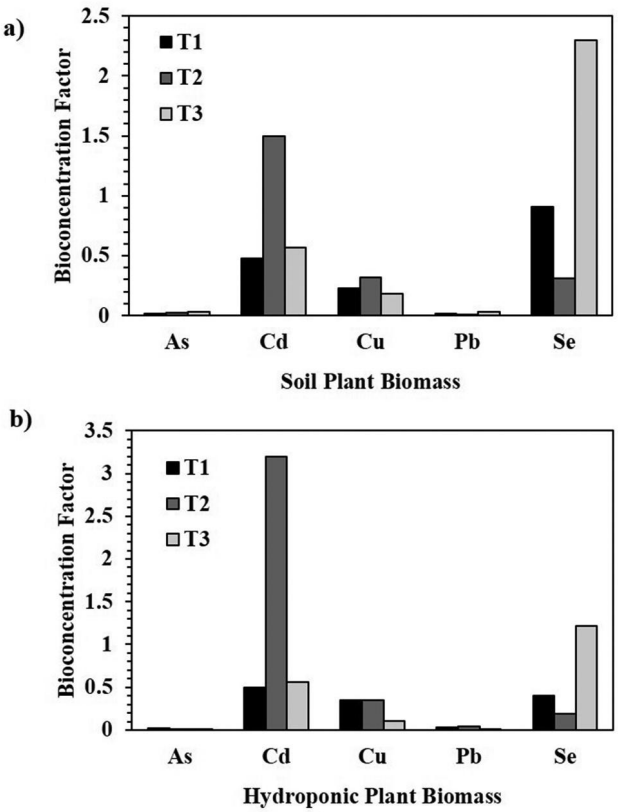

Figure 3. The bioconcentration factor for As, Cd, Cu and Pb in a) the normal soil plant biomass and b) the hydroponic plant biomass. Where T1, T2 and T3 are three samples from different parts of tailing ponds.

average of up to 48% of Se removal was achieved in the soil mix tests, this is potentially because *B. juncea* was reported to be able to remove 13–48% of Se from the contaminated soil [20,29].

A study completed by Lampis et al. (2009) verified that *B. juncea* species (which is said to have a rapid growth) were well capable to accumulate and volatise Se effectively [30]. Volatisation commonly is carried for the volatile forms of a number of inorganic compounds such as selenium [31]. The expected phytoremediation mechanism to take place in order to achieve the removal of Se was phytoextraction. Unlike many heavy metals, Se is more prone to being volatised after being extracted and accumulated [32]. This could be possible because the different phytoremediation technologies are not mutually exclusive, therefore accumulation and volatisation can occur simultaneously [33]. Which justifies the incongruent sum of Se in the soil and that which was accumulated in the plant biomass. Moreover, *B. juncea* also has been reported to have a high capacity to phytovolatise selenium [6].

In this study, further extraction of Se could have been achieved, but the *B. juncea* crops should be harvested as soon as they get matured. This is mostly when they start to germinate so as to avoid unintended secondary germination, for experimental purposes [29]. If not harvested, dried *B. juncea* biomass easily fragments into chip-like particles, that can be easily blown away with the chance of spreading the accumulated heavy metals [29].

Further observations indicate that a general uptake of heavy metals from the tested medium to the shoots. *B. juncea* has been found to have a good ability to transport Cu, Cd, Ni, Pb and Zn from the roots to the shoots, however this excludes selenium [25]. For the given time of the experiments, one of the toxic metals accumulated is copper. Studies have reported 80% of the Cu translocation in the soil for the phytoremediation of heavy metals using *B. juncea* [34]. The low bioconcentration

(<1) of most of these metals shows that they had a low bioavailability to be accumulated up to the shoots of the plants [35].

The experimental results of this study showed that the accumulation of both Cd and Cu in significant concentration, where Cu is said to be influenced the elemental balance in *B. juncea* [36]. Cadmium and lead are more prone to be accumulated in plant biomass due to their high bioavailability [37]. However, this could result in some problems when the treated medium has a polymetallic contamination, which is typical of fly ash or coal washery by-products [38]. The BCF for Cd in the soil mix tests can be regarded as low and high in the hydroponic test when using the scale that [34] adapted for determining the BAF of heavy metals in the soil. According to the scale, a BCF value below 2 is low and above 2 is high [34].

5. Conclusion

In the attempt to extract Se selectively from coal washery, phytoextraction of Se using *B. juncea* achieved a trivial Se removal from the soil. The soil mix-based tests showed a positive uptake of Se, where Se removal account 55% of the initial concentration in the three tailings samples. The hydroponic tests showed a similar trend with 48% of the original Se concentration within the soil in a period of 10 weeks. Several metals were also found to be accumulated within the plant biomass of both tests, and these include As, Cd, Cu and Pb. However, the overall reduction of the Se concentration in the soil is significant despite the trace heavy metal accumulation. Thus, it can be concluded that the phytoextraction of Se using *B. juncea* may be achieved. However, more research effort is needed in future to achieve effective selective removal. Therefore, the present study provides an insight on the practical implications of selective phytoremediation of a heavy metalloid (Se) species from a coal process waste contaminated soil. The study also points out the requirement of more field works in the advancement of phytoremediation of Se in the coal tailing ponds in order to fulfil the selective Se biofortification using *B. juncea*. Based on this study, further studies could be conducted on this topic which may include microbial cultures to the plants system. This could be able to identify the effect of microorganisms on the translocation of the Se from the soil to the shoots of the plants. The study can also be up-scaled; to explore various soil amendment strategies. These may also include the utilisation of regional topsoil to adapt natural conditions of the study area. The experimental work from this study indicates an increased number of replications for the soil substrates would be beneficial. Selection and subjectivation of more samples would allow for extensive randomisation of samples. Furthermore, this would help to account for the varying initial concentrations of the tailing pond samples (study samples). A detailed analysis of the plant biomass can also provide more insights with regards to how the plants behave during phytoextraction. This can also help to provide more information of the distribution of Se within the plant biomass, as well as the total concentrations of the elements within the roots. Further studies could also be conducted to minimise the volatisation of the Se throughout the cultivation period. Moreover, this study can be used as a groundwork for future studies on heavy metal removal from coal washery by-products using phytoremediation.

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Disclosure statement

In accordance with Taylor & Francis and my ethical obligation as a researcher, I hereby declare that there are no conflicts of interest associated with this paper to be disclosed. All results and findings are reported based on the experimental studies that have been conducted for the requirement of a bachelor thesis.

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Publication III

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RESEARCH ARTICLE

Relationships between carboxylate-based nutrient-acquisition strategies, phosphorus-nutritional status and rare earth element accumulation in plants

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Abstract

Background and aims We explored how phosphorus (P) availability influences accumulation of rare earth elements (REE) in plant species with different P-acquisition strategies beyond the commonly explored REE-phosphate precipitation.

Methods Two P-efficient carboxylate-releasing lupin species (*Lupinus albus*, and *L. cosentinii*) and four species with less carboxylate release under P-deficiency (*Triticum aestivum*, *Brassica napus*, *Pisum sativum*, *Cicer arietinum*), were cultivated with a split-root system on two sand types. Phosphorus availability was controlled on one root side by watering the plants with 100 µM P or 0 µM P solutions. Carboxylate release and changes in pH were measured on both sides. Concentrations of nutrients, cadmium (Cd), aluminum (Al), light REE (LREE: La–Eu), and heavy REE (HREE: Gd–Lu, including Y) in roots and shoots were analyzed by ICP-MS.

Results P-deficient *T. aestivum*, *B. napus* and *C. arietinum* did not respond with elevated carboxylate release. These species accumulated more REE when the P supply was low and higher REE concentrations were proportional to declining plant growth. However, *P. sativum*, *L. albus* and *L. cosentinii* accumulated less REE when P supply was low. Plants that strongly acidified the rhizosphere and released low quantities of dicarboxylates accumulated more REE (with higher LREE/HREE ratios) than species that released tricarboxylates.

Conclusion Our findings suggest that REE accumulation strongly depended on rhizosphere acidification, in concert with the amount and composition of carboxylates determining the exclusion of REE-carboxylate complexes. Leaf REE signatures may offer a promising ionomics screening tool for carboxylate release into the rhizosphere.

Keywords Element exclusion · Rhizosphere chemistry · Complexation · Carboxylates

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Introduction

Root carboxylate release is an essential plant strategy to access sparingly-available soil nutrients, especially inorganic phosphate (Pi), iron (Fe), and manganese (Mn) (Shane and Lambers 2005; Lambers 2022). Plants have adapted to conditions of heterogeneously distributed and sparsely-available soil resources and evolved strategies to influence the properties of the soil surrounding their roots (rhizosphere) to create an environment more conducive for nutrient acquisition (rhizosheath). In addition to mutualistic interactions with bacteria and fungi, and alteration of root morphology (Honvault et al. 2021a, b), the most profoundly studied traits involved in direct root–soil interactions include the acidification of the rhizosheath and release of chelating carbon compounds such as carboxylates (Lambers et al. 2015; Honvault et al. 2021b; Lambers 2022). Rhizosphere acidification in the presence of carboxylates increases the solubility and availability of many essential or beneficial elements, including P, Fe, Mn, Cu, Zn and Si, by dissolution, complexation and ligand-exchange reactions (de Tombeur et al. 2021; Lambers 2022). The ability to mobilize Pi and micronutrients in the rhizosphere varies considerably among plant species, functional plant groups (Neumann et al. 2000; Lambers et al. 2013; Lambers et al. 2015) or even genotypes of specific species (Krasilnikoff et al. 2003; Pang et al. 2018). Plant species adapted to P-impoverished or P-sorbing soils, of which Proteaceae and some grain legumes such as *Lupinus albus* have been most profoundly studied, respond to P deficiency by increased release of citrate and malate (Neumann and Römheld 2001; Pearse et al. 2006). In contrast, non-mycorrhizal phosphophilous species in the Brassicaceae, Chenopodiaceae, Urticaceae and some cereals such as *Triticum aestivum*, do not respond to P deficiency with elevated carboxylate release (Pearse et al. 2006; Lambers 2022).

Although carboxylate-based P-acquisition strategies are regulated by plant P status and predominantly target the acquisition of essential mineral nutrients, the resulting chemical changes in the rhizosphere are non-element-specific (Lambers et al. 2015). That means, while nutrient deficiency triggers a shift in metabolism towards elevated proton and carboxylate release, the compounds released solubilize not only nutrients, but also mobilize a number of non-essential elements in the rhizosphere, impacting their chemical speciation and availability to plants as demonstrated for Cd, Pb, Ge and rare earth elements (REEs) (Wenzel 2009; Wiche et al. 2016a, b). In this respect, REEs are particularly interesting to study, because they i) are present in almost all soils at concentrations similar to essential plant nutrients (Reimann et al. 2003; Wiche et al. 2017a), ii) share chemical similarities with essential nutrients, mainly Ca (Tyler 2004; Brioschi et al. 2013), and iii) strongly interact with nutrient-bearing soil minerals (phosphates, Fe-oxyhydroxides), but are neither essential to plants nor strongly toxic (Tyler 2004; Davranche et al. 2017). The REEs comprise a group of 16 elements from the lanthanide series, including lanthanum, yttrium (Y) and scandium (Sc) that are widespread in the earth's crust with concentrations that vary from 66 μg g^{-1} (Ce), 30 μg g^{-1} (La) and 28 μg g^{-1} (Nd) to 0.3 μg g^{-1} (Lu) (McLennan 2001; Davranche et al. 2017). As a unique feature in this group, all 16 REEs exhibit ionic radii similar to Ca^{2+}; however, under most pedologically-relevant conditions, REEs form trivalent cations (Wyttenbach et al. 1998), which strongly interact with phosphate and other negatively charged soil constituents (Diatloff et al. 1999; Cao et al. 2001; Li et al. 2014). In particular, they can form stable complexes with dissolved organic compounds (Pourret et al. 2007; Wiche et al. 2017b), and their stability depends on the nature of the ligand and the REE involved. There are slight differences in ionic radii from light REEs (LREE: La to Eu) to heavy REEs (HREE: Gd to Lu, including Y), leading to differences in their sorption and complexation behavior in soil and their availability in the rhizosphere (Khan et al. 2016; Schwabe et al. 2021; Monei et al. 2022). For REEs in the soil solution, it is generally assumed that uptake of REE^{3+}-ions is mediated mainly by Ca^{2+}-, Na^{+}- and K^{+}-channels (Han et al. 2005; Brioschi et al. 2013), while REE-carboxylate complexes are excluded, relative to free ionic forms (Han et al. 2005; Wiche et al. 2017b). After root sorption, due to the element's higher reactivity, the biogeochemical behavior of REEs in the soil-plant system is not simply analogous to Ca^{2+}, but may resemble that of other trivalent metals, particularly Al^{3+} (Ma and Hirdate 2000). Thus root–shoot transport of REEs depends on their mobility within the plant (Kovarikova et al. 2019), most likely governed by cell-wall absorption, phosphate deposition and intracellular complexation with carboxylates (Ma and Hirdate 2000). Based on the above, plant species that deploy

a carboxylate-based nutrient-acquisition strategy will likely exhibit differences in REE sorption. The P status of the plants and the quantity and composition of the compounds released should influence the processes during the mobilization of non-essential elements and their uptake.

In the present study, we conducted a split-root experiment with two P-efficient carboxylate-releasing lupin species (*Lupinus albus* and *Lupinus cosentinii*) that typically show carboxylate release under low P supply, and four species (*Triticum aestivum*, *Brassica napus*, *Pisum sativum*, *Cicer arietinum*) that lack the ability to respond to P deficiency with elevated carboxylate release (Pearse et al. 2006). A split-root approach was used to exclude the direct effects of P addition on REE availability, i.e. by precipitation as REE-phosphates (Fehlauer et al. 2022; Liu et al. 2022). Thus, one root half received all essential plant nutrients except phosphate, which was supplied to the other root half only. Root carboxylate release and shoot element concentrations (selected nutrients, aluminum, cadmium, REE) were measured, to explore the relation between P nutrition and accumulation of non-essential elements, including total REE uptake and LREE/HREE ratios. If we would be able to show such a correlation, this would offer the possibility to use shoot REE signatures to proxy the involvement of carboxylates in nutrient acquisition.

Methods

Substrates for plant cultivation

In this experiment, 120 pots (7×7×18 cm) were filled with 1.2 kg of sand. Half of the pots (60 pots) were filled with quartz sand (0.1–0.4 mm grain size, 1500 kg m^{-3}), while the other half was filled with a mixture of 75% of quartz sand and 0.25% of river sand (0.4–2 mm grain size, 1320 kg m^{-3}). Here, a second sand type was added to increase the amount of potentially plant-available elements to one half of the split-root systems. The quartz sand had a pH of 5.6 (water/solid 1/10) and 1.1 ± 0.5 mg kg^{-1} calcium lactate-extractable P (van Laak et al. 2018), whereas the mixed sand had a pH of 5.9 and 2.1 ± 0.3 mg kg^{-1} P. In both sand types, the total element concentrations were similar (Table 1); however, the sand types differed regarding the distribution of elements in potentially plant-available element fractions indicated by a sequential extraction analysis considering the distribution of elements in five operationally-defined soil fractions according to Wiche et al. (2017a) (Table 1). In these fractions, the mixed sand was characterized by higher concentrations of P, Mn and Fe (Table 1). Furthermore, the quartz sand showed higher concentrations of mobile/exchangeable and acid-soluble Al and higher concentrations of mobile/exchangeable

Table 1 Total element concentrations and distribution of elements in exchangeable (F1), acid-soluble (F2), oxidizable (F3) and moderately-reducible (F4) fractions (µg g^{-1}) according to Wiche et al. (2017a, b) determined by a sequential extraction method (mean ± sd; $n = 10$)

Substrate	Fraction	P	Mn	Fe	Al	Cd	LREE	HREE
Quartz sand	Total	358 ± 50a	466 ± 245	1773 ± 635	5279 ± 1134	1.44 ± 0.56	12.3 ± 3.4	3.6 ± 0.9
	1	<0.5	0.4 ± 0.1b	0.8 ± 0.2	2.1 ± 0.6a	<0.05	0.40 ± 0.03a	0.10 ± 0.01
	2	<0.5	0.14 ± 0.12b	6.5 ± 1.3b	18 ± 2a	<0.05	0.15 ± 0.03b	0.05 ± 0.01b
	3	123 ± 10b	0.19 ± 0.10b	7.9 ± 2.0b	4.9 ± 0.6b	<0.05	0.10 ± 0.04b	0.21 ± 0.09b
	4	26.2 ± 2.9	0.78 ± 0.09b	89 ± 14	37.8 ± 2.6a	0.09 ± 0.01b	0.60 ± 0.06b	0.17 ± 0.03b
	5	<0.5b	0.64 ± 0.14b	71 ± 31b	73.7 ± 7.2b	0.09 ± 0.01b	0.81 ± 0.13b	0.14 ± 0.03b
Mixed sand	Total	281 ± 36b	334 ± 125	2174 ± 748	5617 ± 1990	1.38 ± 0.34	13.8 ± 1.9	5.0 ± 1.1
	1	<0.5	2.2 ± 0.5a	1.0 ± 0.4	1.4 ± 0.2b	<0.05	0.33 ± 0.04b	0.09 ± 0.01
	2	<0.5	0.71 ± 0.42a	10.3 ± 2.4a	13 ± 3b	<0.05	0.19 ± 0.03a	0.06 ± 0.01a
	3	165 ± 16a	1.87 ± 1.12a	12.3 ± 2.7a	7.6 ± 1.1a	<0.05	0.18 ± 0.05a	0.41 ± 0.17a
	4	23.8 ± 2.6	2.17 ± 0.86a	95 ± 19	32.9 ± 3.1b	0.11 ± 0.01a	0.76 ± 0.08a	0.25 ± 0.04a
	5	8.0 ± 1.7a	4.44 ± 1.69a	518 ± 149a	98.7 ± 14.2a	0.12 ± 0.01a	1.03 ± 0.19a	0.24 ± 0.06a

Differences in means between the two sand types are identified by t-tests with Bonferroni correction. Means with different letters are significantly different at α = 5%

LREE. Thus, in quartz sand, these elements are more easily accessible by roots than in mixed sand. However, Al and both LREE and HREE were generally more concentrated in the mixed sand, especially in the more stable fractions 4 and 5, which were also the significant element-bearing fractions of Cd (Table 1). The LREE / HREE ratios in both sand types were > 1 (Table 1). In particular, quartz sand exhibited a 12% higher LREE / HREE ratio in Fractions 1 (mobile/exchangeable) and a 15% and 33% higher LREE/HREE ratio in Fractions 4 and 5, respectively, where the elements are predominantly bound to amorphous and crystalline structures of oxides and oxide-hydroxides (Table 1). In Fractions 2 and 3, however, the LREE/HREE ratios were similar between the two substrates.

Plant growth

Seeds of *Triticum aestivum* cv Arabella, *Brassica napus* cv Genie, *Pisum sativum* cv Karina, *Cicer arietinum* cv Kabuli, *Lupinus albus* cv Feodora, and *Lupinus cosentinii* cv were surface sterilized by washing the seeds with 0.5% sodium hypochlorite (NaOCl) for 3 min, followed by rinsing with deionized water. Seeds were germinated in Petri dishes in a climate chamber at 20 °C. After germination and development of seminal roots, the seedlings were transferred to a hydroponic culture with a 1/20 strength Hoagland solution (Arnon and Stout 1939), 22 °C room temperature, relative humidity 60% and 600 µmol m^{-2} s^{-2} photosynthetically active radiation. After one week, the primary roots of *B. napus*, *P. sativum*, *C. arietinum*, *L. albus*, and *L. cosentinii* were cut 1 cm below the first lateral roots to obtain a split root system by stimulation of root branching and lateral root development (Saiz-Fernandez et al. 2021). *Triticum aestivum* developed several seminal roots; thus, the abovementioned procedure was unnecessary, and the roots could easily be diverted into different compartments. After cutting, all plants were transferred back into the hydroponic solution and cultivated for another 10 days to allow the plants to recover (Saiz-Fernandez et al. 2021).

Plant individuals with similarly developed root systems were transferred from hydroponic culture into the previously prepared pots filled with sand. Each experimental unit consisted of one plant with a split root system where one part of the root system was placed in a pot with quartz sand and the other part into a pot with mixed sand. The pots were connected with clamps, and the seedlings were stabilized with a stick to support the shoot growing between the two pots. In total, from each plant species, 10 experimental units were prepared. The plants were grown in a growth chamber at 22 °C and 65% humidity, 600 µmol m^{-2} s^{-1} photosynthetically active radiation and watered with a 1/20 strength Hoagland solution containing all essential mineral nutrients, except P. After one week of growth and allowing the plants to extend their roots deeper in the sand substrates, the experimental units were watered with two different nutrient solutions containing either all essential plant nutrients according to a 1/10 strength Hoagland solution except P (P0), or all mineral elements contained in the previous solution with the addition of 100 µM P (P+). Half of the experimental units were watered with P0 solutions at both root sides (50 mL in each pot), whereas the other half received P+ solutions at the root side growing in quartz sand (50 mL) and P0 solutions at the root side growing in mixed sand (50 mL). The addition of treatment solutions was continued every second day over a period of five weeks. Each P treatment was replicated fivefold for each plant species, and the different species and treatments were spatially distributed in a fully randomized design.

Rhizosphere properties and exudate collection

After five weeks, the plants were removed from the sand and carefully shaken to remove loose sand particles. Sand adhering to the root surface was collected by washing the roots with 20 mL of deionized water until 1 g of rhizosheath was obtained. The sand was left in the washing solution for 1 h until the pH was measured using a pH electrode. If necessary, the root was washed a second time without collecting the solution or sand material to remove the remaining sand entirely. The plants were transferred with their individual root systems into a 200 mL sterile Erlenmeyer flasks filled with 100 mL of a 2.5 µM $CaCl_2$ solution. This allowed the collection of root exudates depending on plant species and P-treatment for each root system separately. The plants in the collection solutions were placed back into the growth chamber and allowed to release root exudates over a time period of 3 h. Immediately after the collection, the resulting

solutions were analyzed using ion chromatography. After that, the plants were separated into roots and shoots. Shoots were washed for 1 min with deionized water. The split roots were separately washed for 5 min with ice-cold $CaCl_2$ solution (5 mM) and 1 min with deionized water to remove adsorbed ions from charged root cell structures (Han et al. 2005). Finally, the shoots and roots were dried at 60 °C for 48 h, weighed and stored in centrifuge tubes until being analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

Determination of carboxylates and element concentrations

The dried plant material was ground to a fine powder using a centrifugal mill equipped with a titanium rotor (Retsch ZM 100) and stored in centrifuge tubes. Afterwards, microwave digestion (Ethos plus 2, MLS, Leutkirch, Germany) was carried out with 0.1 g of subsample taken from the ground biomass and measured in duplicate. Samples were mixed with 1.6 mL nitric acid (65% suprapure) and 0.6 mL hydrofluoric acid (4.9% suprapure) and heated to 220 °C in a microwave, according to Krachler et al. (2002). Concentrations of P, Fe, Mn and REEs (Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu) from the diluted digestion solutions and soil solutions were determined by ICP-MS (XSeries 2, Thermo Scientific, Dreieich, Germany) using 10 µg L^{-1} rhodium and rhenium as internal standards. Possible interferences were monitored and corrected if necessary (Pourret et al. 2022).

Concentrations of acetate, fumarate, glutarate, malate and citrate in the collection solutions were determined by ion chromatography equipped with conductivity detection (ICS-5000, 4 mm system, Thermo Scientific, Dreieich, Germany). Inorganic and organic acid anions were separated at 30 °C on an IonPac® AS11-HC column (Thermo Scientific, Dreieich, Germany) using gradient elution with sodium hydroxide as eluent and a flow rate of 1.0 mL min^{-1}.

Data processing and statistical analysis

Concentrations of LREEs and HREEs in the plant and soil samples were calculated as sums of La, Ce, Pr, Nd, Pm, Sm, Eu (LREEs) and Gd, Tb, Y, Ho, Er, Yb, Tm, Lu (HREEs) according to Tyler (2004). Significant differences among means of element concentrations in soil fractions, carboxylate concentrations of P+ and P0 plants, and element concentrations in plant parts cultivated with different P supply were compared by t-test with Bonferroni adjustment of p values using IBM SPSS Statistics 25. Carboxylate release and element concentrations in different root parts of the same plants were compared by a t-test for non-independent samples at $\alpha = 5\%$. Element concentrations, contents and root carboxylate release among plant species within a certain P-treatment were compared by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test. Prior to the analysis, the data were checked for homogeneity of variances using Levene's-test. In case the assumption of homogeneity was violated, the data were log-transformed. If the assumption was still violated, significant differences between means were identified using Welch's ANOVA at $\alpha = 5\%$.

Results

Plant growth and biomass responding to P-supply

The dry biomass varied considerably among the tested plant species (Fig. 1). *Brassica napus* accumulated the most biomass (3.1 g) when P was supplied, and *T. aestivum* accumulated the least biomass when no P was supplied (0.2 g) (Fig. 1A). Phosphorus supply increased the total dry mass (shoot and root mass) of *T. aestivum*, *B. napus*, *C. arietinum* and *L. albus* by 65%, 52%, 27%, and 56%, respectively. In contrast, the total biomass of *P. sativum* and *L. cosentinii* did not respond to P supply (Fig. 1A).

Considering the shoot biomass of plants, the addition of P did not significantly ($\alpha = 5\%$) affect the shoot mass of *P. sativum*, *C. arietinum*, and *L. cosentinii,* and there were no differences among *P. sativum*, *L. cosentinii* and *C. arietinum* (Fig. 1B). However, *L. albus*, *B. napus* and *T. aestivum* strongly responded ($p < 0.01$) to elevated P-supply with 39%, 52%, and 88% greater shoot biomass (Fig. 1B). Considering the whole root system, including both root parts growing in quartz sand and mixed sand, the addition of P did not significantly affect root biomass within a plant species, but tended to increase ($p = 0.14$) total root

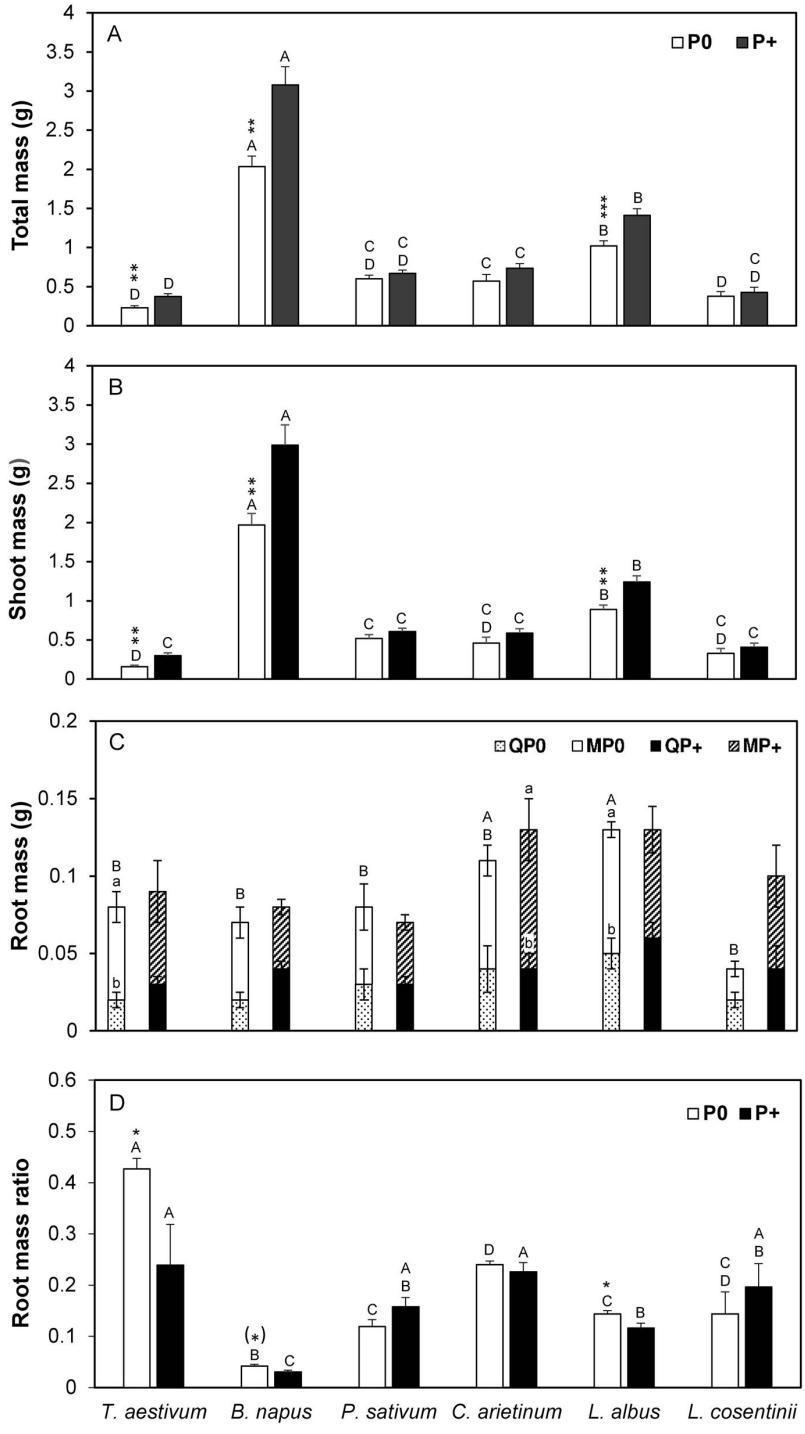


Fig. 1 Shoot mass and root mass (g) of the two root halves growing in quartz sand (Q) and mixed sand (M) and root mass ratio considering both root halves depending on treatment with 100 µM P (P+) and no P (0P). The bars represent means ± se ($n=5$). ANOVA identified differences among species (capital letters) following Tukey's HSD post-hoc test at $\alpha=5\%$. Significant differences in shoot mass and root mass ratios between different P-treatments are indicated by asterisks (**$p<0.01$; *$p<0.05$; (*) $p<0.1$).Differences in root masses between the root halves within a P treatment are indicated with lowercase letters. Capital letters denote differences among the species within a P-treatment. Means with the same letters are not significantly different at $\alpha=5\%$

mass in *L. cosentinii*, by 124% compared with P-deficient plants (Fig. 1C). There were no differences between plant species when the plants were externally supplied with P (Fig. 1C). However, in P0-treated plants, *L. albus* and *C. arietinum* showed the greatest root mass, and the lowest root mass was found for *L. cosentinii* (Fig. 1C).

Considering the development of the split root systems in different sand types, all species tended to have a greater root mass in mixed sand, especially *T. aestivum* and *L. albus,* which showed 200% and 60% more root mass when no P was supplied and *C. arietinum* (125% more biomass in mixed sand) when P was provided. Without the addition of P, the root mass ratio varied significantly among the species showing decreasing ratios from *T. aestivum* > *C. arietinum* > *P. sativum*, *L. albus*, *L. cosentinii* > *B. napus* (Fig. 1D). Addition of P significantly reduced the root/shoot ratio in *T. aestivum*, *B. napus* and *L. albus* by 45%, 25% and 17%, respectively, while in the other plant species, there were no effects (*C. arietinum*) or slightly increasing trends (*P. sativum*, *L. cosentinii*). When the plants received solutions containing 100 µM P, the root mass ratio was similar in *T. aestivum*, *P. sativum*, *C. arietinum* and *L. cosentinii*, but lowest in *B. napus* (Fig. 1D).

Shoot nutrient accumulation

Shoot [P] of plants watered with 100 µM P ranged from 1.21 mg g^{-1} (*B. napus*) to 2.46 mg g^{-1} (*T. aestivum*) (Table 2). *Triticum aestivum* and *L. cosentinii* showed substantially higher [P] in shoots than all other investigated species did. Shoot [P] of *T. aestivum*, *B. napus*, *P. sativum*, *L. albus* and *L. cosentinii* responded to a reduction in P supply by a 57%, 19%, 13%, 12% and 20% decrease of shoot [P], respectively, compared with plants treated with high P (100 µM P). Shoot [P] in *C. arietinum* was almost unchanged; however, under conditions of low P supply, *C. arietinum*, *L. albus* and *L. cosentinii* still displayed the highest shoot [P] compared with *B. napus* and *T. aestivum*.

Concerning the measured micronutrients, *L. albus* exhibited the highest [Mn] and [Fe], irrespective of P-supply. The addition of low P doses tended to decrease shoot [Mn] in *T. aestivum*, *B. napus*, and

Table 2 Concentration of nutrients in shoots of six plant species cultivated under split-root conditions (means ± sd; $n = 5$) and addition of 100 µM P (P+) and no P in the treatment solution (0P)

Species	P-treatment	P mg g^{-1}	Mn µg g^{-1}	Fe
Triticum aestivum	P0	1.06 ± 0.05B	160 ± 35B	122 ± 42A
	P+	2.46 ± 1.03A	206 ± 29B	99 ± 22AB
	p value	0.02	0.06	0.32
Brassica napus	P0	0.98 ± 0.06B	97 ± 17C	39 ± 3B
	P+	1.21 ± 0.12B	114 ± 13 BC	45 ± 8B
	p value	0.05	0.22	0.14
Pisum sativum	P0	1.14 ± 0.09AB	88 ± 18 BC	67 ± 10 BC
	P+	1.31 ± 0.02B	100 ± 36 BC	97 ± 17AB
	p value	0.11	0.50	< 0.01
Cicer arietinum	P0	1.23 ± 0.17A	63 ± 18C	84 ± 31 BC
	P+	1.30 ± 0.11B	53 ± 21C	97 ± 43AB
	p value	0.56	0.42	0.59
Lupinus albus	P0	1.25 ± 0.15A	452 ± 96A	160 ± 71A
	P+	1.42 ± 0.071B	413 ± 51A	107 ± 30A
	p value	0.05	0.25	0.15
Lupinus cosentinii	P0	1.39 ± 0.17A	121 ± 58 BC	88 ± 18 BC
	P+	1.73 ± 0.16A	96 ± 38 BC	87 ± 20AB
	p value	0.03	0.56	0.91
Species	P0	<0.001	< 0.001	< 0.01
	P+	<0.01	<0.001	< 0.01

Capital letters denote significant differences among the species within a P treatment

P. sativum and significantly decreased [Fe] in *P. sativum*. In contrast, shoot [Mn] in low-P plants of *L. albus* and *L. cosentinii* were consistently higher than those in plants that received P with the watering solution (Table 2).

Root nutrient accumulation

Root [P], [Mn] and [Fe] varied substantially among species, P supply and the root part considered (roots growing in quartz sand and mixed sand, respectively) (Table 3). Considering the root part growing in quartz sand, where P-supply was controlled, the addition of low-P solutions (0P) decreased root [P] of all species by 10–25% compared with plants treated with 100 µM P (P+). This effect was strongest in *T. aestivum*, *B. napus*, *P. sativum* and *C. arietinum* ($p < 0.05$) and somewhat weaker in *L. albus* and *L. cosentinii* ($p > 0.05$). When P supply was high, *T. aestivum*, *B. napus*, *C. arietinum* and *L. cosentinii* showed higher root [Mn] than *L. albus*, and the reduction of P supply did not influence [Mn] of roots in quartz sand. However, P-deficient *L. cosentinii* had a 65% higher [Mn] than P-supplied plants ($p = 0.12$). *Triticum aestivum* exhibited the highest [Fe] of all species, irrespective of P treatment, and *B. napus* the lowest. Reduction of P supply increased root [Fe] in *B. napus* and *L. albus* by 161% and 44%, respectively.

In the corresponding mixed sand root part of P-supplied plants, root [P] was the highest in *B. napus* and *P. sativum* and the lowest in *T. aestivum*. When P supply was reduced at the root side in quartz sand, root [P] also declined significantly in the mixed sand root part of *T. aestivum* (19%) and *L. albus* (18%), but it was unchanged in the other species. Considering both P treatments, roots in mixed sand of *T. aestivum* and *C. arietinum* showed consistently lower [P] than roots growing in quartz sand, while in *B. napus* and

Table 3 Nutrient concentrations in roots of six species cultivated under split-root conditions on two sand types, quartz sand and mixed sand (means ± sd; $n = 5$)

Species	treatment	Root part with P supply (quartz sand)			Root part without P supply (mixed sand)		
		P	Mn	Fe	P	Mn	Fe
		mg g^{-1}	µg g^{-1}		mg g^{-1}	µg g^{-1}	
T. aestivum	P0	1.34 ± 0.25aAB	103 ± 28bA	8345 ± 2889aA	0.93 ± 0.13bC	175 ± 49aA	1784 ± 440bA
	P100	1.78 ± 0.23aA	131 ± 27bAB	6270 ± 2032aA	1.15 ± 0.09bC	252 ± 45aA	2614 ± 585bA
	p value	0.02	0.14	0.13	0.01	0.06	0.04
B. napus	P0	1.54 ± 0.19A	148 ± 83A	1243 ± 697B	1.53 ± 0.10A	97 ± 23B	391 ± 97C
	P100	1.79 ± 0.11aA	86 ± 40AB	476 ± 132C	1.56 ± 0.06bA	67 ± 21C	316 ± 48D
	p value	0.03	0.23	0.04	0.65	0.10	0.31
P. sativum	P0	1.52 ± 0.13A	152 ± 109AB	1692 ± 792B	1.39 ± 0.08A	165 ± 71AB	1213 ± 434AB
	P100	1.68 ± 0.04aA	64 ± 35B	1489 ± 34aB	1.35 ± 0.07bB	90 ± 26 BC	1100 ± 89bB
	p value	0.06	0.24	0.68	0.44	0.08	0.63
C. arietinum	P0	1.32 ± 0.09aAB	129 ± 43bA	1515 ± 260aB	1.15 ± 0.06bB	205 ± 30aA	1014 ± 82bB
	P100	1.85 ± 0.30aA	213 ± 126A	1909 ± 799aB	1.21 ± 0.07bBC	133 ± 17B	758 ± 171bC
	p value	<0.01	0.20	0.38	0.15	<0.01	0.02
L. albus	P0	1.10 ± 0.26B	47 ± 8B	1325 ± 250aB	1.08 ± 0.04B	43 ± 8C	632 ± 79bB
	P100	1.35 ± 0.08B	44 ± 14B	919 ± 145aB	1.31 ± 0.17 BC	36 ± 5C	790 ± 109bC
	p value	0.09	0.72	0.01	0.03	0.18	0.25
L. cosentinii	P0	1.38 ± 0.17A	134 ± 23A	2691 ± 2035B	1.20 ± 0.04B	123 ± 77AB	1075 ± 537AB
	P100	1.63 ± 0.45AB	81 ± 53AB	2610 ± 1567B	1.30 ± 0.05 BC	68 ± 21C	1084 ± 344 BC
	p value	0.31	0.12	0.95	0.10	0.10	0.97
Species	P0	0.04	<0.01	0.02	<0.001	<0.001	<0.001
	P100	0.05	0.01	<0.001	< 0.001	<0.001	<0.001

The plants received 100 µM P (P+) or no P (0P) in quartz sand. Capital letters denote differences among plant species within a P treatment, and lowercase letters denote differences between the root halves for a specific element

P. sativum root [P] only differed when P was added to the root half in quartz sand (higher [P] in quartz sand than in mixed sand). *Lupinus albus* and *L. cosentinii* did not show any differences in root [P] between the roots when P was added, nor in situations of P deficiency. Concerning the micronutrients, the root half in mixed sand generally responded more strongly to the P-treatment than the root half growing in quartz sand (Table 3). Specifically, the reduction in P supply increased [Mn] in *B. napus* (45%), *P. sativum* (83%), *C. arietinum* (54%), *L. albus* (20%) and *L. cosentinii* (81%).

Carboxylate release in response to P supply

Considering the quantity of carboxylates released by both root parts per root half and unit of time, *B. napus* released by far the greatest amounts, irrespective of P treatment (Fig. 2A). In *B. napus*, *T. aestivum* and *P. sativum,* the major portion (more than 98%) of the carboxylates released consisted of malate, and citrate was only occasionally detected. In contrast, *C. arietinum*, *L. albus* and *L. cosentinii* released both malate and citrate (Fig. 2A). Carboxylate release was not affected by P supply in *T. aestivum* and *C. arietinum*. *Brassica napus* and *P. sativum* responded to a reduction in P supply with a decrease in carboxylate release by 20% ($p=0.04$) and 44% ($p=0.08$), respectively. In contrast, in *L. albus* and *L. cosentinii*, the reduction of P supply significantly increased total carboxylate release by 159% ($p<0.01$) and 115% ($p=0.03$), respectively, showing an increase of both malate and citrate, but especially of citrate (Fig. 2A). Roots growing in mixed sand released greater amounts of carboxylates per unit time in all tested species, except *B. napus*, which tended to release greater amounts of malate in quartz sand, but only when this root part was supplied with P (Fig. 2B). Also, in the other species, there were significant differences in the response of the different root halves to P supply. *Triticum aestivum* showed no response in any of the root halves (Fig. 2B). *Brassica napus*, *P. sativum* and *C. arietinum* predominantly responded in the root half in quartz sand, where P was added with the watering solution and showed a significant reduction in carboxylate release (24%, 65% and 75%) at low P supply. In comparison, in the root half in mixed sand, carboxylate release in *P. sativum* and *C. arietinum* was unchanged or increased in *B. napus* by 80% when P supply in quartz sand was low. Also, *L. albus* and *L. cosentinii* did not respond in the root half supplied with P but showed an increase of carboxylate release from the root half in mixed sand only. Here, the exudation of malate increased by 121% and 320%, respectively, and citrate release increased by 192% and 870%, respectively, when P-supply was low (Fig. 2B).

Carboxylate release per unit root mass showed far less variation depending on growth substrates and P supply (Fig. 2C). Mixed sand roots still tended to release more carboxylates per unit root mass. However, this trend was only observed in P-supplied *T. aestivum*, *C. arietinum* (irrespective of P-supply) and *L. albus*, but in the latter species only when P was lacking (Fig. 2C). Additionally, in both *C. arietinum* and *L. albus*, exudation rates were affected by P supply, showing less carboxylate release from the root half in quartz sand (*C. arietinum*: 90% decrease) or increasing exudation from the root half in mixed sand (*L. albus*: 105% increase) (Fig. 2C).

Rhizosheath acidification in response to P supply

In the rhizosheath of all plant species and treatments, the pH was significantly higher than that of the unplanted control soil (Fig. 2D). The pH in the mixed sand rhizosphere was consistently higher (on average 0.3 units considering all species) than that in quartz sand as a consequence of the initial pH of the substrates used (Table 1); however, the pH of the substrates was altered depending on plant species, root half and P supply (Fig. 2D). Considering data from both root halves, the rhizosphere pH of P-supplied plants of *B. napus*, *L. albus* and *L. cosentinii* (pH 7.1 ± 0.2) was on average 0.5 units higher than that of *C. arietinum*, *P. sativum* and *T. aestivum* (pH 6.6 ± 0.1). When the P supply was low, the pH in the rhizosheath of *B. napus* was still highest and lowest in the soil of *L. albus* and *L. cosentinii*, which was predominantly driven by a strong acidification at the root half in mixed sand. In contrast, in *P. sativum* the pH was low in both root halves, irrespective of the P treatment.

Considering the different root halves under P+ conditions, the pH in the quartz sand rhizosheath was highest for *B. napus* and showed the pattern *B. napus*>*L. albus*=*L. cosentinii*>*P. sativum*=*T. aestivum*=*C.*

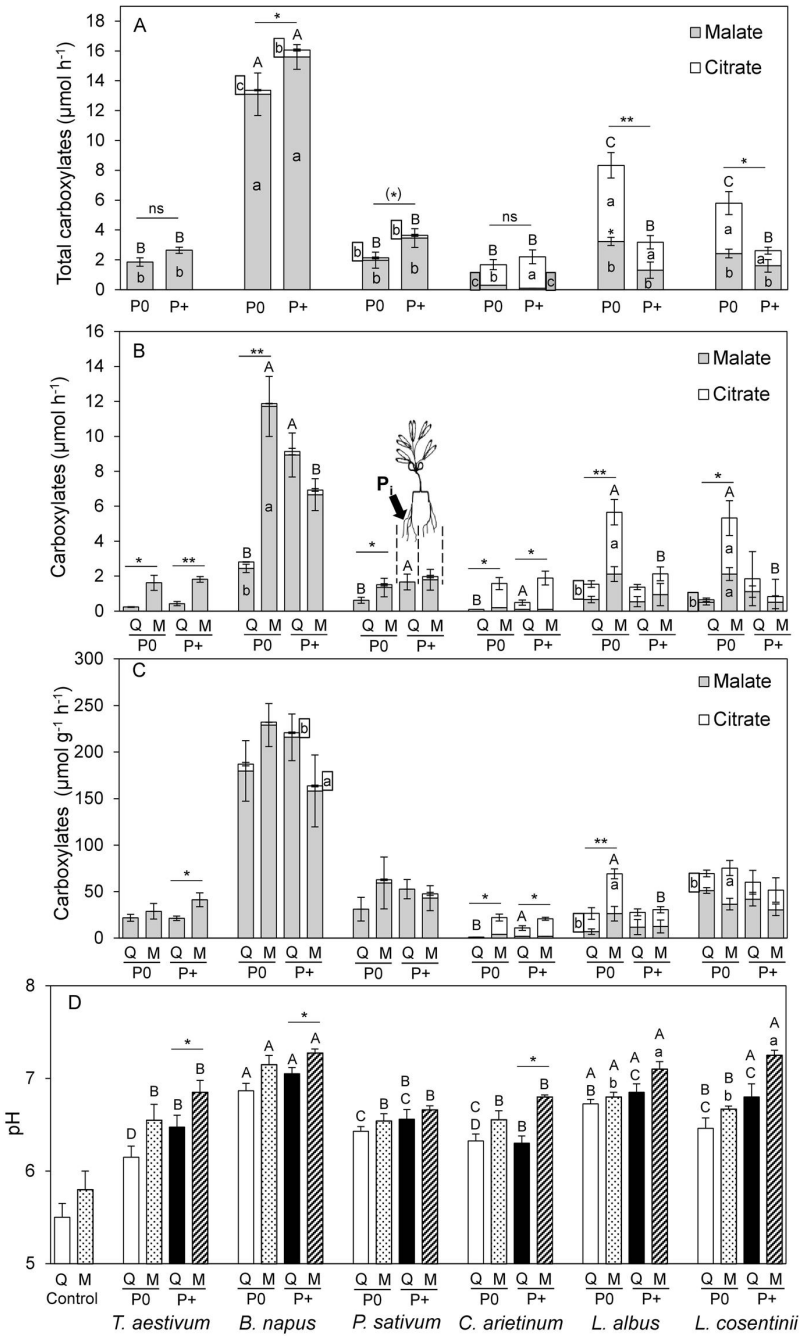


Fig. 2 Total carboxylate release per plant (**A**), (**B**) carboxylate release from root halves growing in quartz sand (Q) and mixed sand (M), (**C**) exudation rates from the different root halves, and (**D**) rhizosphere pH depending on treatment of plants with 100 μM P (P+) or no P (0P) from the root half growing in quartz sand (means ± se, $n=5$). Capital letters indicate significant differences between species within a P treatment. Small letters indicate i) differences among species concerning a specific carboxylate type within a P-treatment (**A**), ii) differences in carboxylate release between different root halves within a P-treatment and species (**B**, **C**) or iii) differences in the pH between the same root half at different P supply rates (**D**). Means with different letters are significantly different at $\alpha=5\%$ identified by Tukey's HSD post-hoc test

arietinum. When P was lacking, the rhizosphere pH of *B. napus*, *C. arietinum* and *T. aestivum* was 0.3 units lower ($p<0.05$) but unchanged (around 6.7±0.2) for *L. albus*, *L. cosentinii* and *P. sativum*. At the root half with mixed sand, the pH of *B. napus, L. albus* and *L. cosentinii* was much (7.2±0.1) higher than that of *T. aestivum*,

C. arietinum and *P. sativum* (6.8 ± 0.1) (α = 1%). Here, the low P supply reduced the pH in the rhizosheath of *L. albus* and *B. napus* by 0.2 units and strongly reduced the pH in the rhizosheath of *L. cosentinii*, by 0.6 units.

Shoot accumulation of non-essential elements

Shoot [Cd] was highest in *B. napus* and lowest in *C. arietinum* (Fig. 3). In contrast, [Al] was the lowest in *B. napus,* and there were no differences among *T. aestivum*, *P. sativum*, *C. arietinum*, *L. albus,* and *L. cosentinii*. Regarding the REE concentrations, *P. sativum* had higher shoot [LREE] and [HREE] than all other species did. HREE concentrations were similar in *T. aestivum*, *B. napus*, *C. arietinum*, *L. albus,* and *L. cosentinii*; however, *B. napus*, *C. arietinum*, and *L. albus* showed higher [LREE] than *T. aestivum* and *L. cosentinii* (Fig. 3).

Phosphorus supply did not significantly affect shoot [Al] and [Cd] in the investigated species, except in *L. albus,* which showed a 61% lower [Cd] when P supply was low. Concomitantly, *L. albus* and *P. sativum* responded with a 42% and 49% decrease of [LREE] and a 42% and 44% decrease of [HREE] at a low P supply. In contrast, *T. aestivum* and *B. napus* exhibited the highest [LREE] in P-deficient plants, and [LREE] was 39% and 19% higher when P supply was high. At the same time, [HREE] were unaffected by P supply in these two species. These changes in [LREE] and [HREE] altered the LREE/HREE ratios of *B. napus* and *P. sativum*, which consistently exhibited higher LREE/HREE ratios in P-deficient plants. In other species, no effects of P addition on the LREE/HREE ratios were observed, except in *L. cosentinii*, which showed the opposite trend with a lower LREE/HREE ratio at a low P supply (Fig. 3).

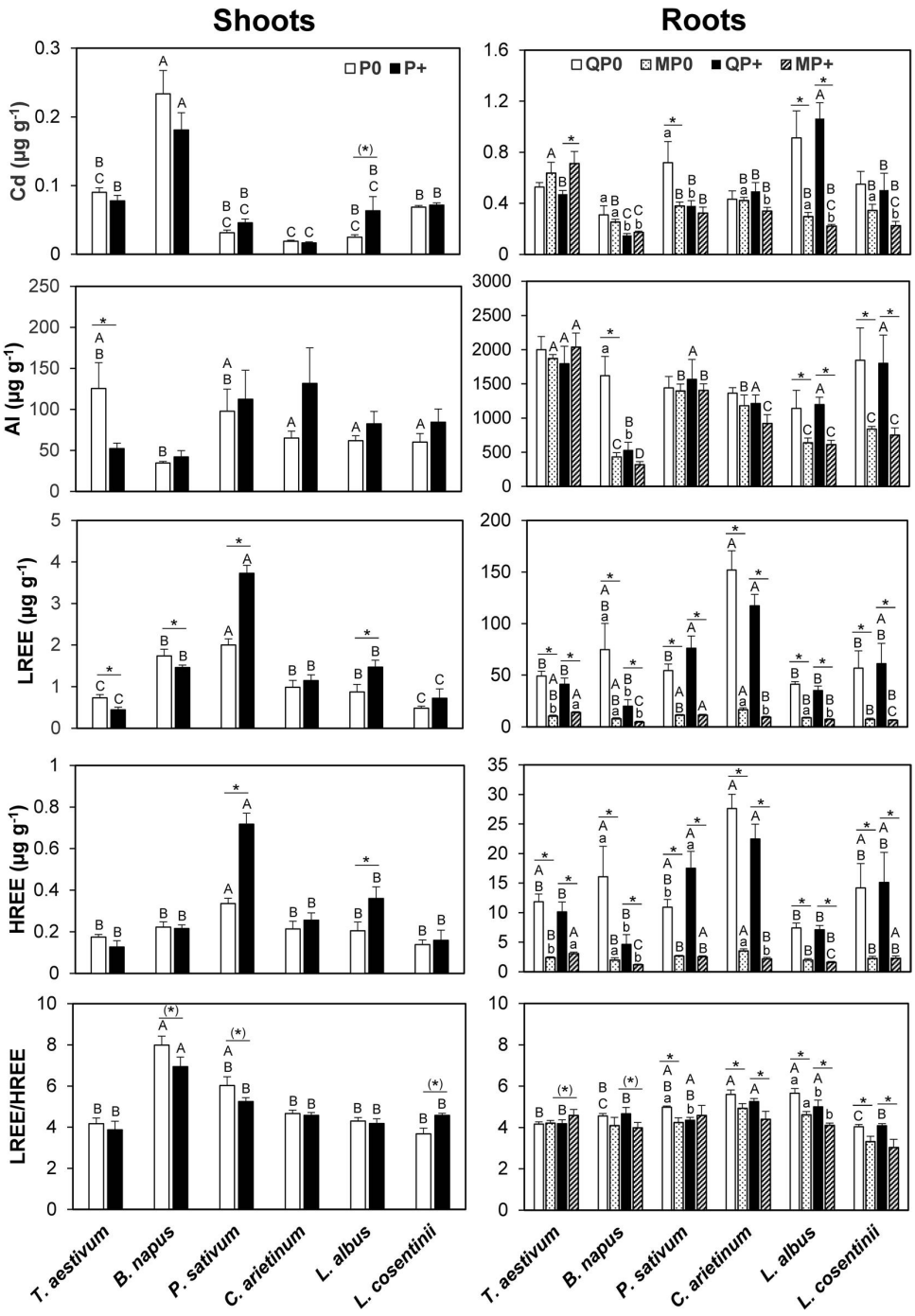
Considering the shoot element contents (calculated as shoot biomass × concentration) (Table 4), *B. napus* showed the highest Cd, Al and REE contents, mainly when the plans were supplied with P and shoot content was lowest in *T. aestivum*. The low P supply did not significantly affect the shoot REE content of *T. aestivum*, *L. cosentinii*, *C. arietinum* and *B. napus*. However, in *L. albus* and *P. sativum,* LREE and HREE contents were 40–46% (*P. sativum*) and 58–60% (*L. albus*) lower at low P supply. Moreover, in *L. albus* Al, Cd contents were 48% and 71% lower. Shoot Al contents in *B. napus* was 47%, lower and Cd content in *T. aestivum* was 43% lower when the P supply was low (Table 4).

Root accumulation of non-essential elements

All investigated species showed significantly higher [LREE] and [HREE] in roots growing in quartz sand, irrespective of the P treatment (Fig. 3). Similarly, quartz sand roots of *L. albus* and *L. cosentinii* exhibited higher [Al] and [Cd]. Considering the different P supplies, quartz sand roots of *T. aestivum*, *C. arietinum, L. albus* and *L. cosentinii* did not show differences in their Al, Cd, LREE and HREE concentrations. However, in *B. napus*, the concentrations of all elements were 102% (Cd), 208% (Al), 275% (LREE) and 248% (HREE) higher in P-deficient roots than in roots supplied with P. In *P. sativum*, P deficiency also increased [Cd] by 89% and decreased [HREE] by 38% but it did not affect [Al] and [LREE]. In the corresponding mixed sand roots, P-supply did not alter [Al] in any of the investigated species and [Cd] was unchanged in *T. aestivum* and *P. sativum*. However, P-deficient *B. napus*, *C. arietinum*, *L. albus* and *L. cosentinii* showed higher [Cd]. Additionally, low P supply increased [LREE] and [HREE] in *C. arietinum* (80% and 59% increase) and *B. napus* (66% and 69% increase) and [LREE], but not [HREE] in *L. albus* (30% increase). In contrast, in *T. aestivum,* [LREE] and [HREE] were 25% and 20% lower at low P supply.

Considering data from both root parts and P treatments, the calculated LREE/HREE ratios of *L. albus* and *C. arietinum* were substantially higher than those in the other species (on average 4.9–5.0 times) and the lowest ratios were found in *L. cosentinii* (LREE/HREE = 3.6 ± 0.7). The LREE/HREE ratios were higher in roots grown in quartz sand than in those in mixed sand, except for *T. aestivum* and *P. sativum*. In *T. aestivum*, the ratios were higher in roots grown in mixed sand of P-supplied plants than in corresponding roots grown in quartz sand but without differences between the P+ and P0 treatments (Fig. 3). In contrast, in *P. sativum*, adding P to the quartz sand decreased the ratio from 5.0 to 4.3. Similarly, in *L. albus*, P addition decreased the LREE/HREE ratios in both root halves (0.7 units in quartz sand and 0.5 units in mixed sand).

Considering the root element contents (Table 5), P-supplied quartz sand roots of *C. arietinum* and *P. sativum* accumulated the greatest amounts of REE and *T. aestivum* and *L. cosentinii* the lowest. In the other root half growing in mixed sand, there were


Shoots
Roots
P0
P+
QP0
MP0
QP+
MP+
Cd (µg g-1)
Al (µg g-1)
LREE (µg g-1)
HREE (µg g-1)
LREE/HREE
T. aestivum
B. napus
P. sativum
C. arietinum
L. albus
L. cosentinii

◂**Fig. 3** Concentrations of trace elements in shoots (left) and roots (right) of split-root plants treated without phosphorus (low P) or with 100 µM P at the root half growing in quartz sand. LREE = sum of La – Eu, HREE = sum of Gd – Lu plus Y (means ± sd, $n = 5$). Differences between the P treatments were identified by t-tests with Bonferroni correction. In shoots, asterisks indicate significant differences between P treatments and means with the same capital letters were not significantly different (identified by ANOVA and Tukey's HSD post-hoc test) among plant species within a P-treatment at $\alpha = 5\%$. Capital letters indicate differences among plant species within a specific root half and P treatment. Lowercase letters denote differences between P treatments within a species and the root side. Additionally, for roots, asterisks indicate significant differences between root sides within a specific P treatment ($\alpha = 5\%$)

no differences in element contents between the species, except for *T. aestivum* and *C. arietinum,* which showed a 3–4 times greater Cd content than *B. napus*, *P. sativum* and *L. cosentinii* did. A low P supply at the root half in quartz sand did not change the contents of Al and Cd in any of the investigated species, neither in quartz sand nor in mixed sand roots. However, in roots grown in quartz sand, LREE and HREE contents were 45% lower in *P. sativum*. Conversely, in mixed sand roots, a low P supply did not change LREE and HREE contents in *P. sativum* and did not affect LREE in the other species. However, in *B. napus*, the low P supply tended to increase the content of HREE by 83%, while in *L. cosentinii* the HREE content decreased by 44%.

Normalized REE pattern in shoots and responses to P supply

The substrate-normalized [REE] calculated for shoots treated with different P levels showed clear differences among the species and partly depended on the treatment with P (Fig. 4). In all plant species, the normalized REE concentrations were $<<1$, and the pattern was generally similar among the species tested with curves downward from left to right showing LREE-enrichment and HREE-depletion.

In *B. napus* and *P. sativum*, the normalized [LREE] relative to [HREE] was much higher than those in *T. aestivum*, *C. arietinum*, *L. albus* and *L. cosentinii* showing LREE/HREE >1. Moreover, *B. napus* and *P. sativum* exhibited steeper curves than *T. aestivum*, *C. arietinum*, *L. albus* and *L. cosentinii* did. Concerning the effects of P addition, *C. arietinum* did not show any differences in the REE pattern between P-supplied and P-deficient plants. When the plants were cultivated under P-deficient conditions, *T. aestivum* and *B. napus* displayed higher normalized [REE], particularly [LREE] and middle-mass [REE] (Gd–Er). In contrast, *P. sativum*, *L. albus* and *L. cosentinii*, showed significantly higher [LREE] and [HREE] when the plants were supplied with P in the nutrient solution.

Discussion

Plant growth, root biomass and nutritional status

In the present experiment, cultivation of plants with split roots growing in different sand types allowed us to control the P supply to one root half without influencing REE availability directly through the precipitation of Al and REE with phosphate in the presence of P. The treatment with low P supply showed less production of shoot and total plant biomass of *T. aestivum*, *B. napus*, and *L. albus,* whereas there was no effect on *C. arietinum*, *P. sativum* and *L. cosentinii* (Fig. 1). The latter species showed virtually unchanged shoot [P] following P addition (Table 2). Shoot [P] did not exceed the concentration that is adequate for crop growth of 2 mg P g^{-1} dry weight (Marschner 1995), except in *T. aestivum*. This was unexpected, given that the plants received a high supply of P (100 µM P as KH_2PO_4) in the nutrient solution. It is possible that a considerable amount of P sorbed onto Al and Fe oxides and hydroxides of the acidic quartz sand. Additionally, after five weeks of plant growth, all plants entered the reproduction phase, so P remobilization to the seeds may have contributed to the low shoot [P] (El Mazlouzi et al. 2020). Shoot [Fe] and [Mn] were largely unchanged at the low P supply. However, lower concentrations of Mn and Fe in P-deficient *T. aestivum* and *P. sativum* (Table 2) might indicate a reduced uptake and/or translocation capacity (Fan et al. 2021). Root [P] was higher in all species in the high P-half. This was not only observed in the root half in contact with the nutrient solution, but also in the other root half, grown in mixed sand of *T. aestivum*, *C. arietinum* and *L. albus*, and to some extent in roots of *L. cosentinii* (Fig. 3). Conversely, [P] of roots grown in mixed sand of *B. napus* and *P. sativum* were unaffected. Indeed, the [P] was highly influenced by root growth.

Table 4 Contents of non-essential elements in shoots of six species cultivated under addition of 100 µM P (P+) or no P (0P) (means ± sd; $n = 5$)

Species	Treatment	Shoot element contents			
		Al	Cd	LREE	HREE
		µg	ng	µg	µg
Triticum aestivum	P0	14 ± 3B	13 ± 4B	0.11 ± 0.02C	0.03 ± 0.01C
	P100	20 ± 12B	23 ± 5 BC	0.14 ± 0.08C	0.04 ± 0.02C
	p value	0.36	0.05	0.52	0.31
Brassica napus	P0	68 ± 10A	526 ± 125A	3.4 ± 0.9A	0.44 ± 0.15A
	P100	128 ± 62A	451 ± 121A	4.3 ± 1.0A	0.64 ± 0.18A
	p value	0.03	0.36	0.19	0.12
Pisum sativum	P0	59 ± 36AB	20 ± 7B	1.24 ± 0.33B	0.21 ± 0.04B
	P100	55 ± 32AB	24 ± 5 BC	2.05 ± 0.34B	0.39 ± 0.05B
	p value	0.35	0.41	0.02	<0.01
Cicer arietinum	P0	31 ± 15B	9 ± 4B	0.46 ± 0.24 BC	0.10 ± 0.06 BC
	P100	76 ± 59AB	10 ± 3C	0.70 ± 0.29C	0.16 ± 0.08C
	p value	0.17	0.72	0.18	0.21
Lupinus albus	P0	56 ± 18AB	23 ± 9B	0.77 ± 0.39B	0.18 ± 0.09B
	P100	107 ± 56A	79 ± 55B	1.82 ± 0.55B	0.45 ± 0.12B
	p value	0.06	0.04	0.01	0.04
Lupinus cosentinii	P0	22 ± 14B	23 ± 10B	0.26 ± 0.16C	0.05 ± 0.02C
	P100	33 ± 22AB	26 ± 9 BC	0.17 ± 0.07C	0.06 ± 0.04C
	p value	0.42	0.65	0.25	0.58
Species	P0	<0.01	< 0.001	< 0.001	< 0.001
	P100	< 0.01	< 0.001	< 0.001	< 0.001

Capital letters denote differences among the species within a P treatment. Differences in element contents between species within a P treatment were identified by ANOVA followed by Tukey's HSD post-hoc test. Differences in element contents in a species between P treatments were identified by t-tests with Bonferroni correction

However, all plants developed more root biomass in the mixed sand. Hence, these findings indicate that the plants allocated a large portion of P absorbed in quartz sand to the other root half growing in mixed sand. The increased root mass ratios of *T. aestivum*, *B. napus* and *L. albus* in P0 treatments (Fig. 1) indicate a relatively increased allocation of dry matter to roots and adjustment of root growth to a low P supply (de Bang et al. 2020). This growth adjustment is determined by the overall nutrient status of the plants (Robinson 1996), and, therefore, might explain the high biomass allocation in *T. aestivum*, which showed the largest differences in shoot [P] resulting from differences in P supply. In contrast, *L. cosentinii*, *C. arietinum* and *P. sativum* did not respond to differences in P supply with altered root mass ratios. These species presumably relied more heavily on chemical changes in the rhizosphere than on more extensive root systems (Pearse et al. 2006). In the present experiment, the mixed sand (roots without P supply) was characterized by a higher pH and higher P availability (Table 1). Therefore, in the present experiment, resource allocation must be considered not only between shoot and roots but also between the different root halves (Fig. 1), allowing us to explore the capacity to respond to nutrient availability by plasticity in root development. Indeed, when P supply was low in quartz sand (P0), all species (except *L. cosentinii*) developed more extensive roots in the mixed sand where the plants were exposed to conditions that allowed them to acquire more nutrients. The P0 treatment reduced the root growth of *B. napus* in quartz sand, but did not affect the root mass of other species at this root side.

When the P supply was higher at the root side in quartz sand, the root mass of *L. albus* was unaffected in mixed sand, but *B. napus* showed a lower root mass. In contrast, *L. cosentinii* and *C. arietinum* had a higher root mass in mixed sand (Fig. 1) when the plants were supplied with P to the roots in quartz sand. This suggests that under P deficiency, the phosphophile *B. napus* mainly relies on readily-available P sources and effectively adjusts its root growth to the compartment where P can be most easily acquired. In contrast, *C. arietinum*, *L. albus* and especially

Table 5 Contents of light rare earth elements (LREE), heavy rare earth elements (HREE) in roots of six species cultivated under split-root conditions on two sand types, quartz sand and mixed sand, respectively (means ± sd; $n = 5$)

Species	P-supply	Root half with P supply (quartz sand)				Root half without P supply (mixed sand)			
		LREE	HREE	Cd	Al	LREE	HREE	Cd	Al
		µg	µg	ng	µg	µg	µg	ng	µg
T. aestivum	P0	0.57 ± 0.23B	0.13 ± 0.05	6.4 ± 3.5b	23 ± 8b	0.56 ± 0.08B	0.13 ± 0.03AB	34 ± 6aA	106 ± 32aA
	P100	0.81 ± 0.42C	0.19 ± 0.09 BC	9.4 ± 5.3bBC	34 ± 14B	0.83 ± 0.74	0.17 ± 0.12	36 ± 23aA	126 ± 124
	p value	0.28	0.26	0.32	0.18	0.18	0.54	0.81	0.87
B. napus	P0	1.44 ± 1.32AB	0.30 ± 0.26	5.2 ± 4.1b	24 ± 20	0.42 ± 0.15B	0.11 ± 0.04AB	13 ± 2Ba	24 ± 9B
	P100	1.01 ± 0.59aBC	0.23 ± 0.15aBC	7.6 ± 2.4B	21 ± 10B	0.26 ± 0.08b	0.06 ± 0.02b	8 ± 2B	15 ± 5
	p value	0.53	0.34	0.82	0.34	0.82	0.07	0.84	0.11
P. sativum	P0	1.7 ± 1.0aAB	0.34 ± 0.21a	18 ± 9	38 ± 20	0.53 ± 0.27bBC	0.13 ± 0.07bAB	17 ± 10AB	59 ± 27AB
	P100	3.1 ± 0.2aAB	0.62 ± 0.05aAB	14 ± 1 BC	55 ± 8AB	0.51 ± 0.17b	0.11 ± 0.05b	15 ± 8AB	64 ± 28
	p value	0.04	0.06	0.47	0.15	0.51	0.74	0.74	0.80
C. arietinum	P0	4.6 ± 4.1A	0.84 ± 0.76	15 ± 6b	62 ± 49	1.17 ± 0.55A	0.25 ± 0.11A	29 ± 8Aa	82 ± 35AB
	P100	4.7 ± 1.1aA	0.86 ± 0.23aA	18 ± 4B	50 ± 27AB	0.94 ± 0.62b	0.22 ± 0.13b	31 ± 10A	91 ± 64
	p value	0.96	0.97	0.38	0.68	0.68	0.73	0.81	0.78
L. albus	P0	1.8 ± 1.2aAB	0.32 ± 0.21	40 ± 36	59 ± 44	0.65 ± 0.16bAB	0.15 ± 0.03A	24 ± 6A	51 ± 7AB
	P100	2.2 ± 0.6aB	0.44 ± 0.11aB	64 ± 8aA	75 ± 19aA	0.76 ± 0.32b	0.13 ± 0.02b	21 ± 9bAB	48 ± 14b
	p value	0.53	0.26	0.14	0.41	0.41	0.54	0.53	0.50
L. cosentinii	P0	0.86 ± 0.39aAB	0.22 ± 0.11a	13 ± 10	31 ± 15	0.15 ± 0.08bC	0.05 ± 0.02bB	6.0 ± 1.9B	17 ± 10B
	P100	0.79 ± 0.29aC	0.19 ± 0.07aC	8 ± 5 BC	32 ± 26B	0.30 ± 0.17b	0.09 ± 0.04b	8.9 ± 3.7B	30 ± 13
	p value	0.76	0.72	0.52	0.94	0.19	0.07	0.86	0.21
Species	P0	0.03	0.06	0.06	0.35	< 0.01	0.04	<0.001	< 0.01
p value	P100	<0.001	<0.001	<0.001	<0.01	0.12	0.08	<0.01	0.48

The plants received 100 µM P (P+) or no P (0P) in quartz sand. Capital letters denote differences among the plant species within a P-treatment, and lowercase letters denote differences between the root halves

L. cosentinii appeared to follow a more conservative strategy and sustained root development in the mixed sand with higher total nutrient concentrations, increasing the chance to maintain P and micronutrient supply through changes in rhizosphere chemistry.

Modifications of rhizosphere chemistry in response to P supply

After five weeks of plant growth, all species tested had entered the reproductive phase and started flowering. So the carboxylate release observed in the present study may not necessarily characterize the plant's nutrient-acquisition efficiency, because carboxylate release typically declines when plants enter the reproductive stage (Mimmo et al. 2011). However, the observed exudation rates among P-supplied and P-deficient plants can be used to characterize the species' general response to the P status (Fig. 2). In this study, the amount of carboxylates released from the different root halves per unit time (Fig. 2B) integrates root mass and carboxylate release per unit mass (Fig. 2C). They characterize the species' ability to chemically influence the root environments. In contrast, exudation rates per unit of time and root mass characterize the physiological response to environmental conditions.

T. aestivum, *B. napus*, *P. sativum* and *C. arietinum* did not show differences in rhizosphere pH in response to P supply (Fig. 2D); however, the rhizosheath pH was lowest in *P. sativum* and *C. arietinum* (Fig. 2D), highlighting the capacity of these species to acidify the rhizosphere irrespective of P supply (Pearse et al. 2006). In contrast, *L. albus*, and *L. cosentinii* strongly acidified the rhizosphere when P was lacking in the nutrient solution, especially in the mixed sand.

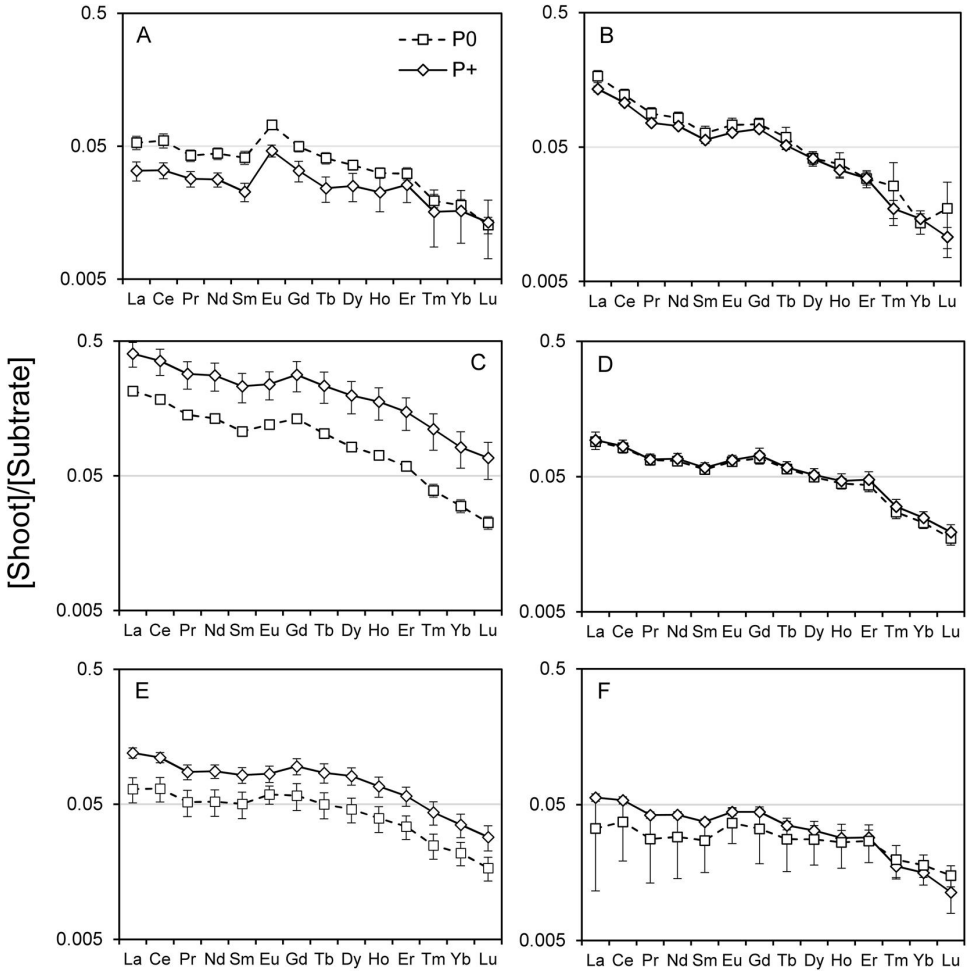


Fig. 4 Substrate-normalized REE patterns (calculated by dividing the shoot concentrations by the average total element concentrations of the sand substrates) in **A**) *Triticum aestivum*, **B**) *Brassica napus*, **C**) *Pisum sativum*, **D**) *Cicer arietinum*, **E**) *Lupinus albus* and **F**) *L. cosentinii* treated with 100 µM P (P+) or no P (0P)

It is generally assumed that the response of plants to nutrient deficiency is determined by the overall nutrient status of the plant, as demonstrated for lupins and some Proteaceae species (Shane et al. 2003a, 2003b; Wang et al. 2013). However, In *C. arietinum,* the production and release of carboxylates appears to be independent of plant P status (Wouterlood et al. 2004) and *B. napus* and *T. aestivum* typically show slow and declining carboxylate release under P-deficient conditions (Pearse et al. 2006). Consistently, in the present study, P deficiency increased the carboxylate release of the lupins, reduced carboxylate release in *B. napus* and *P. sativum*, but did not affect the amount of carboxylates released in *C. arietinum* and *T. aestivum* (Fig. 2A, B).

Besides changes in amounts, the composition of root exudates is an integral factor determining P-mining efficiency (Jones 1998; Lambers 2022). Despite the lower carboxylate release of *B. napus* at a low P supply, *B. napus* released the greatest amounts of carboxylates, mainly malate. Compared with dicarboxylates (malate) released by *T. aestivum*, *B. napus* and *P. sativum*, citrate, a tricarboxylate which was the dominantly

carboxylate released by *C. arietinum, L. albus* and *L. cosentinii* (Fig. 2A) forms more stable complexes with soil cations and consequently is more efficient at releasing P and micronutrients by complexation and ligand exchange reactions (Jones 1998). Moreover, when P supply was low in the quartz sand substrate, all species responded with decreased amounts of carboxylate release at this root side (Fig. 2B) which was primarily due to reduced root mass as a consequence of P starvation (Fig. 1). In contrast, carboxylate-exudation rates were unaffected in P-starved roots of *L. albus* and *L. cosentinii* and these species showed an up-regulation of carboxylate release in roots in mixed sand (Fig. 2B, C) attributing to these species' ability to respond to a low P supply with adjustment of root activity and rhizosphere chemistry.

Accumulation of non-essential elements related to P-supply and carboxylate release

A low P supply may affect the accumulation of non-essential elements through i) altered plant growth and thus an enrichment per unit biomass, ii) altered uptake and translocation when uptake is mediated by nutrient transporters that are affected by the growth-limiting nutrient, and iii) altered solubility and chemical speciation in the rhizosphere determining the accessibility for transport mechanisms. If altered solubility is involved, when the availability is limited by mobility in soil, any increase in solubility following changes in chemical speciation will ultimately increase diffusion towards the root and the probability of the element entering the root. Conversely, when the mobility of elements is high(er), changes in the chemical speciation from the ionic form to a metal-organic complex may decrease availability through exclusion at the site of uptake (Barber and Lee 1974). In the present experiment, all plants altered the rhizosheath pH and released carboxylates depending on species and P supply (Fig. 2). The sand substrates contained the elements in sparingly soluble forms (Table 1). Less than 0.1% of Cd, Fe, Mn, and Al were present in mobile forms (Fraction 1). In contrast, the solubility of REE was somewhat higher, especially in the quartz sand (Table 1). Nonetheless, all species contained detectable concentrations of all elements with high variability among the species tested (Fig. 1). Aluminum and REE showed a similar behavior in the shoots, consistent with the literature (Liu et al. 2021; Fehlauer et al. 2022). In *B. napus*, high shoot and low root [Cd] can be primarily explained by the efficient influx and transport of Cd from roots to shoots (Selvam and Wong 2009).

Concerning the effect of P supply, the REE concentrations in shoots and roots responded more sensitively than those of Al and Cd, given that four out of six species showed significant differences in [LREE] and [HREE] following a reduction of P supply (Fig. 3). Of these species, *C. arietinum* and *T. aestivum* did not respond to altered element accumulation and showed a relatively flat normalized REE pattern with a slight decrease in HREE accumulation (Gd–Lu) (Fig. 4). These species did not respond to a low P supply with altered carboxylate release (Fig. 2). The higher LREE and Al concentrations in roots in mixed sand of P-deficient *C. arietinum* (Fig. 3) corresponded with less root biomass (Fig. 1). Enrichment could largely explain this in the roots which led to unchanged element contents in the plants (Table 3). Thus, the higher concentrations of Al and LREE in shoots and roots of P-deficient *T. aestivum* (Fig. 3) were accompanied by lower biomass production (Fig. 1) and unchanged element amounts accumulated in the plant compartments (Tables 3; 4).

Similar to *T. aestivum*, the shoot biomass of *B. napus* was lower as a consequence of lower P supply (Fig. 1) but without changes in Cd, Al and HREE concentrations (Fig. 3), whereas LREE concentrations were significantly higher (Fig. 3) and Al, LREE, and HREE contents were less. (Table 3). Additionally, in P-deficient plants, total carboxylate release was less (Fig. 2), suggesting that the element pattern in shoots resulted from less element uptake in concert with a preferential root-shoot transfer of LREE relative to HREE and LREE accumulation in shoots. HREE form more stable complexes with low-molecular-weight organic anions, for instance, citrate, during long-distance transport in the xylem (Ma and Hirdate 2000; Yuan et al. 2017). However, based on the higher charge density, HREE are preferentially sorbed onto cell walls during radial transport and form more stable complexes with metabolites released into the rhizosphere. Given that REEs are predominantly taken up in ionic form through Ca, K, and Na channels (Han et al. 2005), carboxylates and other chelating compounds would alter the chemical speciation, and hence the uptake and accumulation of REE, including the ratio of LREE/HREE (Wiche et al. 2017b). Element exclusion through extracellular

complexation has been studied in detail for Al in Al-resistant species (Zheng et al. 1998; Ma et al. 2001; Kochian et al. 2004) and Cd in *L. albus* (Römer et al. 2000). For a specific carboxylate (e.g., citrate), the complex stabilities decrease in the order HREE > LREE > Al > Cd (Byrne and Li 1995; Martell et al. 2004), while for a given element (e.g., La), the complex stabilities decrease in the order citrate > malate > acetate (Fig. 5).

Han et al. (2005) demonstrated that organic acids promote the uptake of La by barley, but the effect of the acid decreased in the order acetic acid > malic acid > citric acid, which can be explained mainly by decreased sorption of La onto the apoplast in the presence of the acid anion but a reduced uptake with increasing complex stability (Han et al. 2005). In the present experiment, *B. napus* released large quantities of malate (Fig. 2), a dicarboxylate with a lower complexation constant (La: log K 4.37) compared with that of citrate (La: log K 7.63). Nonetheless, the large quantities released should favour complex formation and element exclusion, which might also explain the lower total REE concentrations in *B. napus* than in *P. sativum*. *Pisum sativum* released much smaller amounts of dicarboxylates but strongly acidified the rhizosphere (Fig. 2D) and mobilized the elements in plant-available (ionic) forms (Cao et al. 2001; Wiche et al. 2017b). Slight differences in the complexation behavior between LREE and HREE might have influenced the LREE accumulation in this species at a low P supply (Figs. 3 and 4). Indeed, P-deficient roots exposed to quartz sand with higher mobility of REE (Table 1) showed higher concentrations of Al, LREE and HREE but did not affect net root sorption (Table 3) with lower carboxylate release (Fig. 2). Conversely, P-deficient roots in mixed sand released greater amounts of carboxylates (Fig. 2B) and showed higher concentrations (Fig. 3) and element contents (Table 3), most likely through increased element dissolution followed by decreased internal element transport. This contention is supported by the responses in *P. sativum*, *L. albus* and *L. cosentinii*. *Pisum sativum* strongly acidified the rhizosheath in both root parts, irrespective of P supply, and released only small amounts of carboxylates, mainly malate (Fig. 3). A reduction in P supply did not change shoot and root biomass (Fig. 1). Still, it decreased the concentrations and contents (Fig. 3) of LREE, HREE, Fe and Mn with higher LREE/HREE ratios in P-deficient plants. Conversely, in shoots of *L. albus* and *L. cosentinii*, the concentrations and contents of LREE, HREE and Cd declined (Fig. 3) at a low P-supply which was accompanied by greater exudation of citrate (Fig. 2). Although in *L. cosentinii*, this effect was somewhat less pronounced than in *L. albus*, in *L. cosentinii* P-deficient plants displayed significantly lower

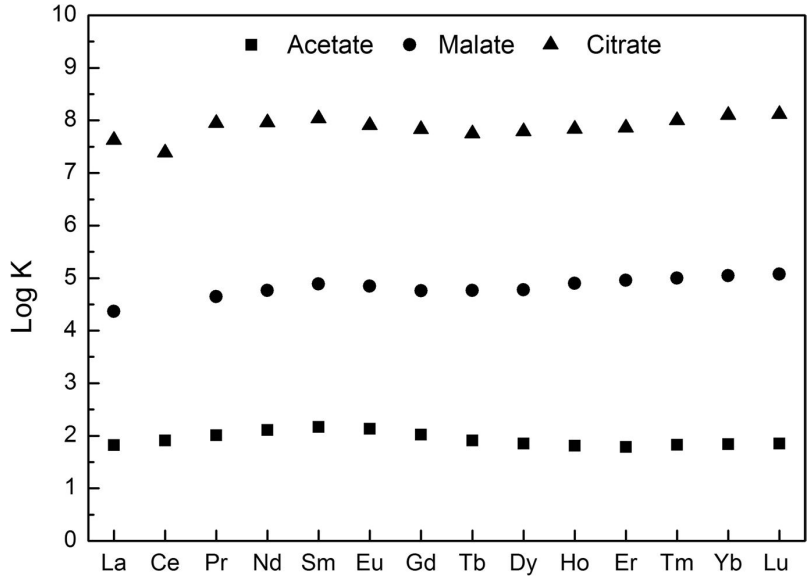


Fig. 5 Stability constants (T = 25 °C or 20 °C) for REE complexation with organic acid anions. Data from Martell et al. (2004)

LREE/HREE ratios indicating a higher HREE translocation relative to LREE when P-supply was low. In contrast, P-deficient roots of *L. albus* showed higher LREE/HREE ratios, irrespective of the root half, while in *L. cosentinii*, the LREE/HREE ratios in roots were unaffected. This can be primarily explained by the strong acidification of the rhizosphere of *L. cosentinii,* shifting the carboxylic acid: carboxylate ratio towards the acid form (Pearse et al. 2006), preventing complex formation and favouring uptake of LREE in *L. albus* but not in *L. cosentinii*. In the latter species the presence of carboxylates might have increased the release and uptake of HREE from sparingly-available element forms from the HREE-enriched mixed sand (Table 1).

Conclusion

We demonstrated that plant P status influenced the accumulation of the non-essential elements Cd, Al, and REE, beyond the commonly recognized mechanism of REE-phosphate precipitation in roots. Plants that strongly acidified the rhizosphere and released small quantities of dicarboxylates accumulated the highest concentrations of REE. Conversely, modest rhizosphere acidification and large amounts of carboxylates were associated with a significantly lower accumulation of REE. Phosphophile species or plants that do not respond to P deficiency (*B. napus*, *T. aestivum*, *C. arietinum*) with increased carboxylate release accumulated REE to higher concentrations when P supply was low, which was explained largely by reduced growth and thus enrichment of the elements in the plant biomass. Additionally, in these species REE-phosphate precipitation might have contributed to a lower REE accumulation in P-supplied plants. In contrast, plants that released more tricarboxylates under conditions of P deficiency accumulated more REE when the P supply was high and carboxylate release was low. The proposed mechanism involves the mobilization of the elements in the rhizosphere through carboxylate and proton release, pH-dependent formation of REE-carboxylate complexes with complex stabilities depending on the amount and composition of carboxylates with HREE-complexes > LREE-complexes and exclusion of the complexes during uptake, radial transport and/or translocation. This suggests a functional overlap of carboxylate-based belowground traits related to P nutrition and exclusion of REE, which otherwise might become toxic in REE-enriched growth environments. The relationship between plant nutrition and REE accumulation could also explain the large variability in REE accumulation among different plant species and plant individuals growing in the same soil. The proposed model provides a mechanistic explanation for the REE-hyperaccumulation in Proteaceae (Van der Ent et al. 2023) and highlights the potential of leaf REE signatures to characterize plant species regarding their P-acquisition strategy through changes in rhizosphere chemistry following an ionomic approach.

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Data availability All data obtained during the experiment are contained in the manuscript.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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Publication IV

Okoroafor, P. U., Mann, L., Amin Ngu, K., Zaffar, N., **Monei, N. L.**, Boldt, C., Reitz, T., et al. (2022). Impact of Soil Inoculation with Bacillus amyloliquefaciens FZB42 on the Phytoaccumulation of Germanium, Rare Earth Elements, and Potentially Toxic Elements. *Plants*, *11*(3), 341. MDPI AG. doi: 10.3390/plants11030341

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Impact of Soil Inoculation with *Bacillus amyloliquefaciens* FZB42 on the Phytoaccumulation of Germanium, Rare Earth Elements, and Potentially Toxic Elements

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Abstract: Bioaugmentation promises benefits for agricultural production as well as for remediation and phytomining approaches. Thus, this study investigated the effect of soil inoculation with the commercially available product RhizoVital®42, which contains *Bacillus amyloliquefaciens* FZB42, on nutrient uptake and plant biomass production as well as on the phytoaccumulation of potentially toxic elements, germanium, and rare earth elements (REEs). *Zea mays* and *Fagopyrum esculentum* were selected as model plants, and after harvest, the element uptake was compared between plants grown on inoculated versus reference soil. The results indicate an enrichment of *B. amyloliquefaciens* in inoculated soils as well as no significant impact on the inherent bacterial community composition. For *F. esculentum*, inoculation increased the accumulation of most nutrients and As, Cu, Pb, Co, and REEs (significant for Ca, Cu, and Co with 40%, 2042%, and 383%, respectively), while it slightly decreased the uptake of Ge, Cr, and Fe. For *Z. mays*, soil inoculation decreased the accumulation of Cr, Pb, Co, Ge, and REEs (significant for Co with 57%) but showed an insignificant increased uptake of Cu, As, and nutrient elements. Summarily, the results suggest that bioaugmentation with *B. amyloliquefaciens* is safe and has the potential to enhance/reduce the phytoaccumulation of some elements and the effects of inoculation are plant specific.

Keywords: *Bacillus amyloliquefaciens*; phytoextraction; potentially toxic elements; germanium; rare earth elements; bioinoculants

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1. Introduction

Soil pollution majorly arises from the dumping of waste from natural or anthropogenic sources in soil, thereby causing undesirable impacts on the chemical, biological, and physical properties of air, soil, and water [1]. In addition, the study of trace elements in the environment has drawn much attention to the presence of critical raw materials (CRMs) like germanium (Ge), rare earth elements (REEs), and potentially toxic elements (PTEs) in different kinds of waste and combustion products. Some of these elements are widely dispersed in soils and do not exist in concentrated deposits [2–7].

The environmental presence of these elements of interest has implications that are either negative or positive, depending on their concentration and the sensitivity of the living organisms in the environment. Potentially toxic elements and some CRMs have negative consequences on living organisms when they exist in concentrations that are beyond permissible limits, as has been revealed by some studies [8,9]. Their effect on

biochemical reactions in living organisms can impact metabolic processes and reduce crop yields [1]. Thus, there is a need for remediating the environment when these PTEs exist in toxic concentrations. In addition, the presence of CRMs in soils and various depositories such as waste implies that there is the possibility of element recovery via urban mining to increase the supply of CRMs since the economic development of these CRMs, despite the increasing demand and price, has not been sustainable [1,6,7,10,11].

Phytoextraction is among the several techniques that can be used to remediate the high presence of PTEs in soil and biologically extract CRMs (phytoremediation for PTEs and phytomining for CRMs). It is cost effective and has less environmental impact [12]. It involves the use of plants to sequester elements from the soil via the roots [13]. However, phytoextraction can be limited by a low availability of elements in the soil for uptake and low plant biomass production. This is because some elements may not be available in chemical species readily available for plant uptake as they exist in different soil fractions of potentially mobile element forms bound to clays, minerals, and oxides of iron and manganese, which has a strong influence on their behavior in soil and availability for phytoextraction. One example is iron (Fe), which exists as iron hydroxide in soil. The hydroxide is solubilized by bacteria to free the iron ion or the iron is solubilized by siderophore released by some soil bacteria, as reported by Schwabe [14]. These bacteria impact the solubility by changing the speciation of the element of interest in the rhizosphere, hence the plethora of studies that are targeted towards understanding the chemical behavior and bioavailability of these elements of interest in soil and enhancing the process of phytoextracting them from soil [10,13,15–18].

The improvement of soil health and the bioavailability of elements can be done via bioaugmentation using soil microbes [18]. The bioavailability of elements greatly determines the success and long-term sustainability of phytomining and phytoremediation, implying that bioaugmentation with associated plant growth-promoting rhizobacteria (PGPR) could enhance the efficacy of phytoextraction [19]. Plant growth-promoting rhizobacteria form a kind of beneficial symbiotic association with plants where the plant exudates serve as a carbon source for bacteria [13]. They enhance element mobility and bioavailability through several mechanisms, such as the secretion of chelating agents—such as siderophores, phenolic compounds, and organic acids—as well as inducing the acidification or redox changes in the plant rhizosphere [17]. Thus, they augment the capacity of plants for the remediation of contaminated soil and the reduction of the phytotoxicity of PTEs.

In addition, many studies have reported these PGPR strains as being capable of solubilizing phosphate in soil, including a recent one by Schwabe et al. [14]. However, the strains are outnumbered by other bacteria that are easily established in the rhizosphere such that they cannot compete favorably. This limits the amount of P solubilized and the expression of other beneficial mechanisms through which these bacteria influence element bioavailability and plant growth. Therefore, to maximize the benefit of the plant growth-promoting traits of these bacteria, the inoculation of plants or soil by higher concentrations of bacteria than those usually found in soils is required [20]. Some of these PGPRs have been produced at a commercial scale as microbial formulations are used in agriculture as microbial inoculants in soil bioaugmentation [21].

Several studies have demonstrated the involvement of beneficial micro-organisms, such as rhizobacteria or endophytes associated with plant roots, for the extraction or accumulation of elements of interest or for reducing toxicity and the immobilization of elements in soil [13]. *Pseudomonas maltophilia* was reported to have reduced the toxicity of chromium (Cr) in soils by reducing the toxic Cr^{6+} to nontoxic and immobile Cr^{3+} and to have restricted the mobility of toxic ions like cadmium (Cd^{2+}), lead (Pb^{2+}), and mercury (Hg^{2+}) [13,22,23]. Rajkumar and Freitas [24] also observed that the inoculation of *Ricinus communis* with *Pseudomonas sp*. PsM6 or *P. jessenii* PjM15 increased plant biomass production and enhanced the phytoextraction efficacy for nickel (Ni), copper (Cu), and zinc (Zn) by the production of indole-3-acetic acid (IAA) and solubilizing phosphate. *Bacillus amyloliquefaciens* BSL16 was

reported to increase Cu accumulation and the growth of rice seeds and tomato plants under Cu stress [25]. Furthermore, Abou-Shanab et al. [26] reported the possibility of an increase in Ni accumulation by rhizobacteria. *Bacillus lichenformis* was reported to have enhanced the accumulation of Cu, Cd, Pb, and Cr [27]. In addition, a recent study by Kabeer et al. [28] reported a reduced shoot content of Cu and Pb upon treatment with rhizobacteria, while Schwabe et al. [14] reported an increased shoot content of Ge and REEs upon inoculation with PGPR.

These studies have highlighted the roles that PGPR plays in plant element accumulation. However, to the best of our knowledge, the effects of bioaugmentation by *B. amyloliquefaciens* FZB42 inoculated via the commercially available formulation RhizoVital®42 on the simultaneous uptake of PTEs, CRMs such as Ge and REEs, nutrients, shoot yield, and bacterial community composition using *Fagopyrum esculentum* cv *Moench* and *Zea mays* cv *Badischer Gelber* as test plants and for the purpose of phytomining and phytoremediation have not been studied. Therefore, the main aim of this study was to evaluate the effects of bioaugmentation using inoculum from a commercially produced microbial formulation of *B. amyloliquefaciens* FZB42 on the phytoextraction of PTEs (arsenic (As), lead (Pb), cobalt (Co), copper (Cu)) and CRMs (germanium (Ge), and the sum total of REEs (REET)), as well as iron (Fe), silicon (Si), calcium (Ca), and phosphorus (P)—regarded as the nutrient elements in this study—from soil. We hypothesized that the inoculation of soil with Rhizovital 42 (bioformulated *B. amyloliquefaciens* FZB42) inoculum will enrich the strain in soil, and improve plant shoot yield and the aboveground phytoaccumulation of elements, given the reports of the effects of PGPR on element accumulation from previous studies.

2. Results

2.1. Effect of Inoculation on Soil Microbial Community Composition and B. amyloliquefaciens Abundance in Soil

The analyses of the bacterial community at the end of the experiment revealed no significant differences between the studied treatments. Neither the crop nor the application of Rhizovital showed significant effects on the relative abundance of main bacterial phyla (Figure 1A, Table 1) or on the community composition (Figure 1B). At the phylum level, Actinobacteriota predominated all soil communities (with a mean of 28%, Figure 1A, Table 1), followed by Proteobacteria (18.4%), Acidobacteriota (10.1%), Chloroflexi (7.8%), Firmicutes (7.3%), and Planctomycetota (7.2%). Although the principal coordinates analysis (PCoA) indicated dissimilarities between the bacterial communities (Figure 1B), these differences were not related to the applied treatments, indicating that the inoculated strain did not affect the inherent soil community.

Regarding the investigated target strain *Bacillus amyloliquefaciens* FZB42, the results of Illumina sequencing show that compared to reference soils for both plants, soils inoculated with *B. amyloliquefaciens* generated a lower number of sequences (*F. esculentum* = 61,553, *Z. mays* = 50,967) than uninoculated soils (*F. esculentum* = 62,317, *Z. mays* = 55,217) and had a lower number of operational taxonomic units (OTUs) (*F. esculentum* = 1641, *Z. mays* = 1567) than inoculated soils (*F. esculentum* = 1718, *Z. mays* = 1570). In addition, the results show that soils in which *F. esculentum* was grown generated a higher number of sequences and had higher OTU numbers compared to the soils planted with *Z. mays*. For *F. esculentum*, inoculated soils generated 764 and 77 fewer sequences and OTUs, respectively, than uninoculated soils, while for *Z. mays*, soils inoculated with PGPR generated 4250 and 3 fewer sequences and OTUs, respectively, than uninoculated soils. In reference soils in which *F. esculentum* was grown, no sequences related to the inoculated strain were found, whereas in soils inoculated with the PGPR, approximately 510 sequences were generated. Similar observations were found for the reference soils (four sequences generated from just a single replicate) versus inoculated soils (383 sequences generated) in which *Z. mays* was grown. Therefore, the results demonstrate that the strain *B. amyloliquefaciens* was present in the inoculated soils with average relative abundances of 0.85% and 0.75% for the bacterial soil communities of *F. esculentum* and *Z. mays*, respectively.

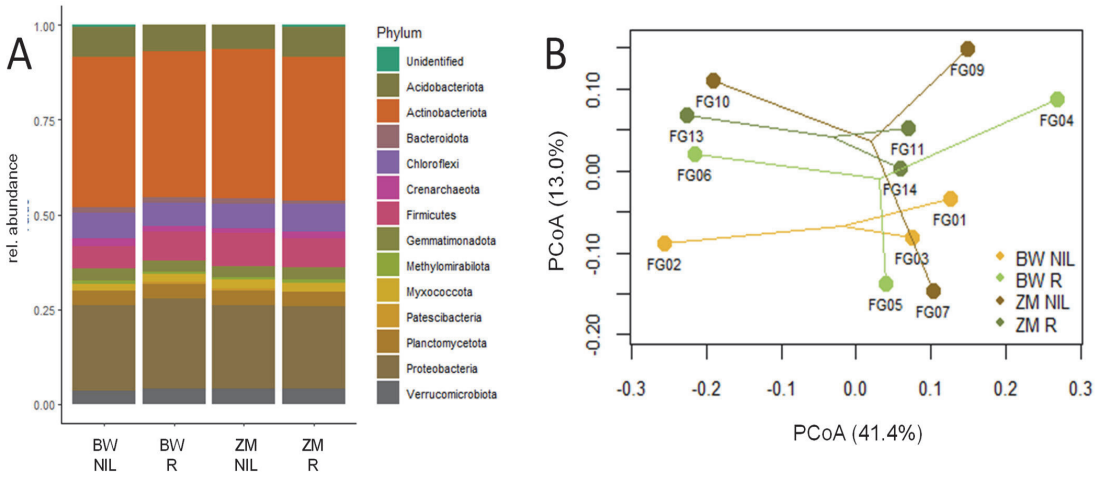

Figure 1. Bacterial community composition in the plant rhizosphere at plant harvest. (**A**) Bar plot showing the average distribution of main phyla (with abundances of >0.5%) in the soils. (**B**) Visualization of a multidimensional scaling approach (PCoA) to explore dissimilarities between the soil communities. The respective three replicates of each color-coded treatment are connected to each other. ZM = maize (*Z. mays*), BW = buckwheat (*F. esculentum*), NIL = reference soil, R = inoculated soil, FGxx = sample ID.

Table 1. Mean proportions (given in % of the total community) of main phyla (with abundances of >0.5%) in the soils of the studied treatments. Soils were cultivated with *Fagopyrum esculentum*/buckwheat (BW) or *Zea mays* (ZM) without inoculation (NIL) and with inoculation (R) of *B. amyloliquefaciens*.

Phylum	BW NIL	BW R	ZM NIL	ZM R
Acidobacteriota	10.31	9.81	9.83	10.53
Actinobacteriota	28.98	27.88	27.62	27.39
Bacteroidota	2.83	3.08	2.57	2.21
Chloroflexi	7.97	7.56	7.65	8.00
Crenarchaeota	0.59	0.58	0.61	0.63
Firmicutes	6.67	7.55	7.69	7.09
Gemmatimonadota	4.03	4.31	4.35	4.57
Methylomirabilota	0.74	0.50	0.66	0.74
Myxococcota	3.11	3.33	3.72	3.94
Patescibacteria	1.39	1.66	1.61	1.67
Planctomycetota	7.26	7.58	7.14	6.95
Proteobacteria	18.40	18.37	18.73	18.05
Verrucomicrobiota	2.74	2.65	2.64	2.89
Unidentified	0.72	0.81	0.64	0.79

2.2. Effect of PGPR on Shoot Yield and Accumulation of Investigated Elements

For both *Z. mays* and *F. esculentum*, there were no significant differences between the biomass produced by plants grown on reference soils and soils inoculated with *B. amyloliquefaciens*. Inoculation with PGPR only slightly affected the shoot yield of *F. esculentum* and *Z. mays*. Inoculated plants showed an 8% higher shoot yield for *F. esculentum* and an 18% higher yield for *Z. mays* compared to the reference plants (Figure 2). For *Z. mays*, inoculation with *B. amyloliquefaciens* FZB42 did not significantly alter the accumulation of nutrient elements, Ge, REET, and most PTEs considered in this study except Co, for which there was a significant decrease in accumulation of 57% (Figure 3). Contrastingly, the inoculated plants displayed slight increases of 10% and 23% in the shoot contents of Cu and As, respectively.

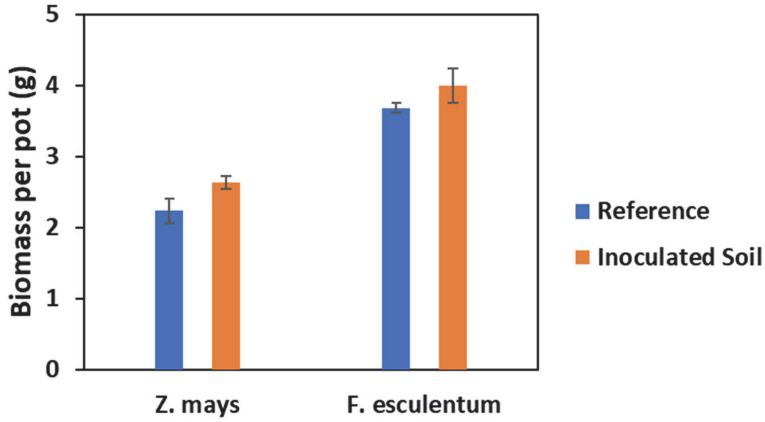

Figure 2. Effect of inoculation on shoot yield of *Zea mays* and *Fagopyrum esculentum* (mean ± SE, $n = 3$).

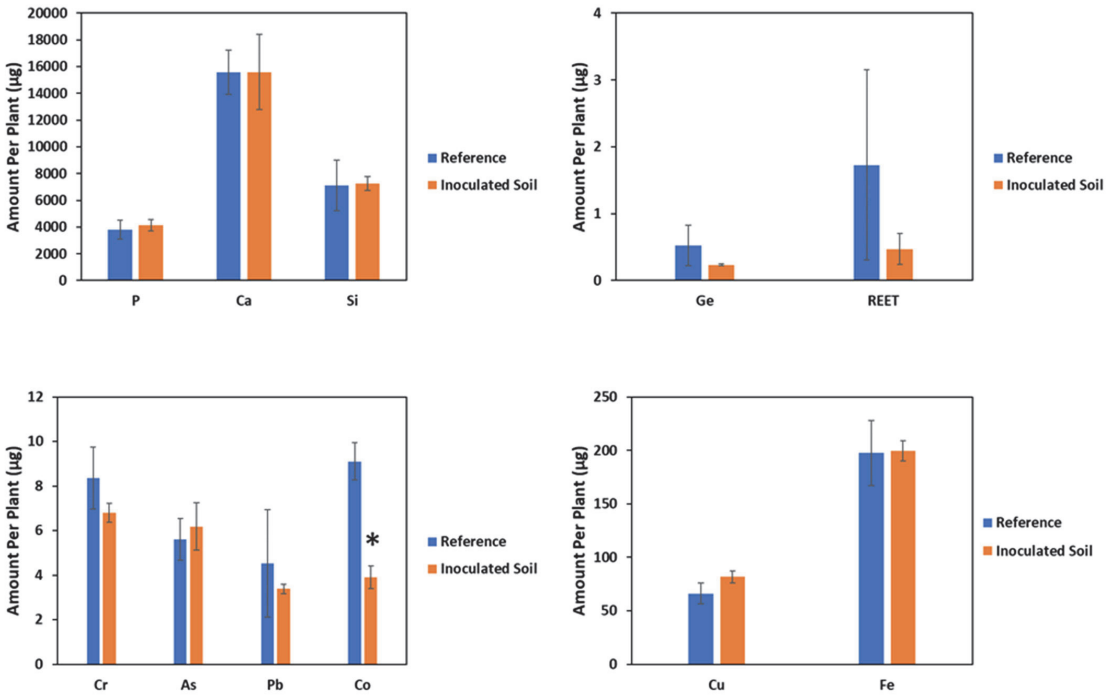

Figure 3. Effect of inoculation on phytoaccumulation of investigated elements by *Zea mays*. Significant difference ($p \leq 0.1$) between means indicated by asterisk * (mean ± SE, $n = 3$).

In addition, in *Z. mays*, concentrations (Tables 2 and 3) of the most investigated elements decreased by percentages between 6% and 75%, with the exception of Cu, which was not affected. For *F. esculentum* growing on inoculated soils, the shoot contents of Cr, Fe, and Ge decreased by 59%, 15%, and 40% respectively, while the accumulation of the rest elements was not significantly impacted except for Ca, Cu, and Co, for which there were significant increases of 40%, 383%, and 2042%, respectively (Figure 4). In addition, observations for the effect of inoculation on the concentrations of the investigated elements

in *F. esculentum* (Tables 2 and 3) were similar to the observations for the effects of inoculation on the shoot contents of the investigated elements.

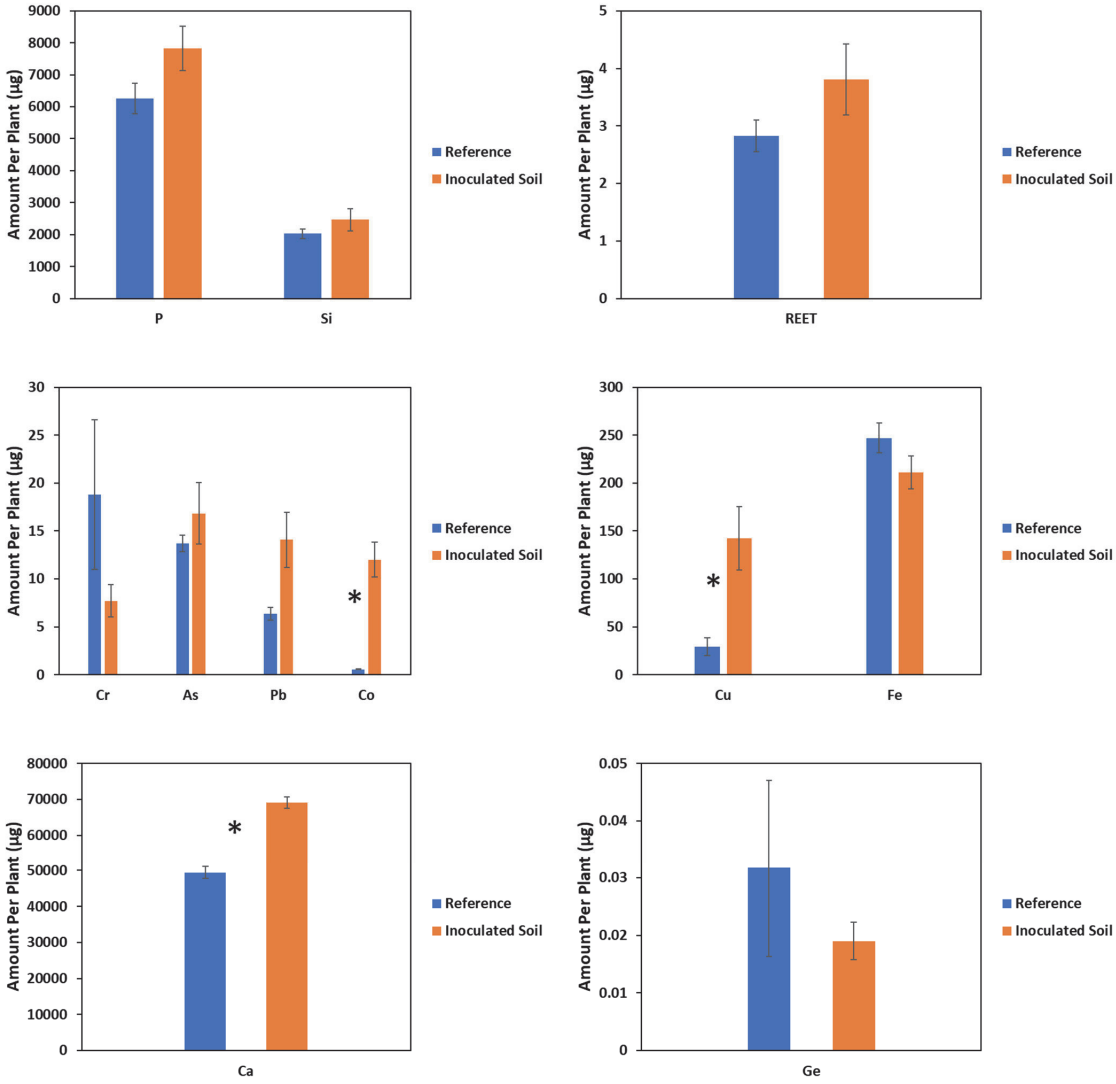

Figure 4. Effect of inoculation on phytoaccumulation of investigated elements by *Fagopyrum esculentum*. Significant difference ($p \leq 0.1$) between means indicated by asterisk * (mean ± SE, $n = 3$).

Table 2. Effect of soil inoculation on concentration (µg/g) of PTEs, Ge, and REET in shoots of test plant species.

Species	Treatment	Cr	As	Pb	Co	Cu	Ge	REET
Z. mays	**NIL**	3.86 ± 0.90	2.50 ± 0.31	1.93 ± 0.89	4.14 ± 0.51	30.1 ± 5.74	0.26 ± 0.16	0.68 ± 0.54
	R	2.58 ± 0.11	2.34 ± 0.38	1.28 ± 0.05	1.48 ± 0.18	31 ± 1.52	0.09 ± 0.004	0.17 ± 0.08
	Statistic [a]	1.97	0.10	0.52	24.0	0.019	1.05	0.87
	***p* value**	0.29	0.77	0.55	0.03	0.9	0.41	0.45
F. esculentum	**NIL**	5.15 ± 2.22	3.72 ± 0.18	1.72 ± 0.16	0.15 ± 0.01	7.94 ± 2.49	0.01 ± 0.004	0.77 ± 0.06
	R	1.89 ± 0.34	4.14 ± 0.58	3.49 ± 0.58	2.97 ± 0.30	36.1± 8.90	0.005 ± 0.001	0.96 ± 0.17
	Statistic [a]	2.11	0.47	8.68	90.98	9.25	0.81	1.21
	***p* value**	0.28	0.55	0.08	0.011	0.078	0.46	0.37

Mean ± SE, *n* = 3, NIL = reference, R = inoculated soil. Statistic [a] means asymptotically distributed F statistic for Welch's ANOVA.

Table 3. Effect of soil inoculation on concentration (µg/g) of selected nutrients in shoots of test plant species.

Species	Treatment	P	Ca	Si	Fe
Z. mays	**NIL**	1681 ± 181	6981 ± 611	3137 ± 636	88 ± 8
	R	1578 ± 208	5975 ± 1162	2744 ± 142	76 ± 6
	Statistic [a]	0.14	0.59	0.36	1.28
	***p* value**	0.728	0.499	0.603	0.327
F. esculentum	**NIL**	1699 ± 122	13,434 ± 692	549 ± 34	67 ± 4
	R	1953 ± 94	17,421 ± 1294	611 ± 53	53 ± 4
	Statistic [a]	2.73	7.39	0.95	6.06
	***p* value**	0.18	0.07	0.39	0.07

Mean ± SE, ***n*** = 3, **NIL** = reference, **R** = inoculated soil. Statistic [a] means asymptotically distributed F statistic for Welch's ANOVA.

3. Discussion

3.1. Effects of Inoculation on Root Colonization, Rhizosphere Bacterial Communities, Nutrient Supply, and Plant Growth

Important aspects for the application of PGPR inoculation-assisted plant biomass production and phytoremediation include the establishment of the inoculant in the soil as well as the effect of the inoculant on the existing microbial community. This is important because it has been reported that bacterial communities in soils are often resistant to the introduction of foreign species [29], which could hinder the establishment and effectiveness of the inoculant [30]. In addition, inoculants could be invasive and alter the existing soil microbial community composition [31], although the success of an invasion is dependent on the diversity of the existing microbial community [32]. Thus, we assessed the relative abundance of *B. amyloliquefaciens* in the soil community and checked for differences between the bacterial community composition in the soils. The results of this study, which show that the strain established itself in the soil community with a relative abundance of approximately 1%, indicate a successful integration of the strain into the bacterial community. The high abundance of the inoculated strain in the soil indicates that the existing microbial community did not prevent the establishment of the strain in the soil. This finding could be related to the fact that *Bacillus* species are known to produce endospores that help them survive and establish themselves in soil [27,31]. In addition, a possible restricted niche overlap in the soil between *B. amyloliquefaciens* and the resident bacteria, which is sometimes influenced by a variation in nutrient demands and spatial separation, may have contributed to the establishment of *B. amyloliquefaciens* in the soil. In addition, the results of the PCoA, which show that inoculation did not cause a significant shift in the bacterial

community composition, agree with the findings of Chowdhury et al. [33], who reported that *B. amyloliquefaciens* FZB42 did not significantly impact the indigenous rhizosphere bacterial community. Niche processes, which are determined by plant selection power and other environmental factors, such as soil chemistry, are the major factors driving microbial community assemblage in the rhizosphere [34–36]. The absence of a significant shift in the microbial community composition suggests that inoculation with *B. amyloliquefaciens* did not impact plant selection power or other environmental factors enough to cause a significant shift in the niche processes within the soil microbial community. This alleviates the fears that the inoculation of soil with *B. amyloliquefaciens* may significantly disturb the structure of the microbial community and the fear that *B. amyloliquefaciens* will not survive in soil when used as an inoculant, confirming that they are safe for use in agriculture and phytoremediation purposes.

3.2. Effects of Inoculation on Shoot Yield

In this study, we used fertile PTE-polluted soil from the post-mining area of Freiberg. Thus, it was not surprising that the biomass production (shoot yield) was only slightly affected by inoculation under the conditions of adequate nutrient supply, as evident in the slight increase in the biomass of the inoculated plants compared to the non-inoculated reference plants. This slight increase, although insignificant, could be due to the plant growth-promoting properties of *B. amyloliquefaciens* related to the secretion of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid deaminase (ACC deaminase) activity, some of which promote increased photosynthetic rates [37–40]. Stefan et al. [41] reported increased photosynthetic rates in runner bean upon inoculation with two PGPRs, stating the IAA-producing ability of the bacteria as a possible cause. Similarly, Naveed et al. [42] reported enhanced shoot biomass production and physiology (photosynthesis, chlorophyll content, and efficiency of photosystem II) in *Z. mays* upon inoculation with endophytic PGPR, which colonized the plants. In addition, an increased acquisition of nutrients may have contributed to the slight increase in the biomass observed, but this would be mostly true for *F. esculentum*, where inoculation increased the accumulation of most nutrients (P, Si, and Ca) between 22% and 25% compared to *Z. mays*, where the slight percentage increase upon inoculation was not more than 8%. The increased accumulation of nutrients might be a result of a *B. amyloliquefaciens*-induced increase in the nutrient element solubilization and the mobility of these nutrients in the rhizosphere, thus making these elements bioavailable for plant uptake. A *Bacillus* species was reported by Jamil et al. [43] to have increased Ca and P accumulation in plants, and this is in tandem with the results of our study. The reduced accumulation of Fe, despite *B. amyloliquefaciens* being a siderophore-producing bacterium, may be because the siderophore produced under the conditions in the substrate favored the solubility and binding of metals other than Fe, hence the decrease in the accumulation of Fe [44].

3.3. Effects of Inoculation on PTE and CRM Accumulation

The effect of *B. amyloliquefaciens* on plant growth is of interest for plant growth promotion in agriculture and biomass production for bioenergy purposes, especially on marginal soils characterized by high concentrations of PTEs. However, beyond these reasons, there is interest in the effects of *B. amyloliquefaciens* on the phytoextraction of elements from soil, for example, PTEs [45] and CRMs such as Ge and REEs.

In this study, the observed effects of inoculation on element accumulation by *F. esculentum* (a forb and strategy 1 plant with respect to Fe acquisition) and *Z. mays* (a grass and strategy 2 plant with respect to Fe acquisition) differed for some elements and were similar for others. These differences in the observed effects may be related to the plant species' characteristics, such as growth habits, element acquisition strategy, and colonization of the plant roots by bacteria [17]. In addition, although the effects of many elements on accumulation by both test plants upon inoculation were substantial, these effects were statistically insignificant for most elements, possibly due to variation in the extent of inoculation effects among plant

replicates. Plants were placed in a randomized manner under the light source, causing differences in intensity of light exposure among replicates. These differences can affect the photosynthetic and transpiration rates among plant replicates, which could have an effect on the extent inoculation affects plant replicates. Only the effects of inoculation on Ca and Cu phytoextracted by *F. esculentum* and Co phytoextracted by both test plants were significant. The increased accumulation of Cu and As in *Z. mays*, as well as Cu, As, Co, and REET in *F. esculentum* upon inoculation with *B. amyloliquefaciens* may be connected with the solubilization of these elements by substances produced by the bacteria, such as carboxylic acids, indole acetic acids, and siderophores, as well as root exudates produced by plants, which solubilize these metals and facilitate their uptake by the plant roots [13]. The formation of siderophore–metal complexes and the release of elements from organic matter decomposition by bacteria, which can be taken up directly by plants, increases the accumulation of metals in plants [17,46]. These results agree with those of Khan et al. [25], who reported that *Bacillus amyloliquefaciens* BSL16 increased the accumulation of Cu in rice and stated the production of organic acids, biosurfactants, and siderophores as possible reasons for the increased Cu accumulation, as suggested by Sheng et al. [47]. Additionally in agreement with our results are those from the study of Lampis et al. [48], who reported a 22% increase in As accumulation upon plant inoculation with PGPR, crediting the increase to the combined effect of the beneficial properties of siderophore and IAA production by the PGPR, as well as the reduction of arsenate to arsenite.

The contrasting results of the decreased accumulations of Cr, Pb, Co, Ge, and REET in *Z. mays*, as well as of Cr and Ge in *F. esculentum* may be due to a possible immobilization of these elements in the soil upon inoculation with bacteria, thus limiting uptake by *Z. mays*. It is possible that *B. amyloliquefaciens* used polymeric substances, exopolysaccharides that are capable of forming biofilms around plant roots, and other chemical substances, such as some carboxylates it produces to immobilize these elements by forming stable complexes with their ions in the soil solution, thus limiting their uptake by plants [27,49–51]. Ashraf et al. [52] reported the formation of soil sheaths in the root zone of wheat to limit the flow of toxic ions into wheat roots upon inoculation with exopolysaccharide producing *Bacillus* spp. Fan et al. [53] reported that the expression of genes involved in the formation of biofilms was enhanced by maize root exudates. Silva et al. [54] reported that the inoculation of *Z. mays* with some PGPR strains reduced the accumulation of Cr in *Z. mays*, and this reduction in the accumulation of Cr may be due to the reduction of the mobile Cr^{6+} to the immobile toxic Cr^{3+} ions, as reported by Jing et al. [13]. This agrees with the results of our study and suggests that reductions in the oxidation states of element ions in the soil, which lead to element immobilization and reduced bioavailability, might be the reason for the reduced uptake of some elements upon inoculation with PGPR. However, some studies have reported a decrease in As accumulation in plants upon inoculation with PGPR, including *Bacillus* [51,55].

Furthermore, element accumulation patterns upon inoculation may have been due to chemical relationships or similarities in origin that resulted in simultaneous accumulation by plants, as the plant may not have easily taken them up differentially or, in some cases, because of competition for the same transport channels or sites. For example, the observed higher accumulation of As and P in *Z. mays* upon inoculation may be connected to the chemical relationship between As and P [56]. In addition, Ge and Cr are usually bound to silicates [6,57,58] and, as such, it may be that the increased accumulation of Si was a result of preferential accumulation of Si over Ge and Cr. Other examples could be Pb and P [59], P and Ca [60], Ca and REET [61].

Conclusively, our study has highlighted the possibilities of enhanced biomass production and phytoextraction of elements, including nutrients, PTEs, and elements of economic value, using *Z. mays* and *F. esculentum* as test plants and commercially available *B. amyloliquefaciens* FZB42 bioformulation as the inoculant. We demonstrated that it is possible that upon inoculation of soil with bacteria, biomass production by *Z. mays* and *F. esculentum* can be enhanced, while phytoextraction can be enhanced or impeded depend-

ing on several interacting factors related to plant species characteristics, such as growth habits, element acquisition strategy, and the colonization of plants by bacteria, which could differ between the two plant species [17]. In addition, the study highlights that the use of commercially available microbial inoculant containing *B. amyloliquefaciens* FZB42 as the PGPR, as well as for phytoremediation purposes, is safe, as the *B. amyloliquefaciens* FZB42 establishes itself well in soil and does not majorly affect the structure of the indigenous soil microbial composition. Although the above-mentioned effects of inoculation might not all be significant, we think that they are meaningful, as they indicate what possibilities of element accumulation there could be upon the inoculation of soils in which *F. esculentum* and *Z. mays* are grown, using *B. amyloliquefaciens* as the microbial inoculant. Thus, the findings of this study may provide useful information when planning agricultural projects that intend to use microbes to boost plant growth and nutrient content, for environmental remediation projects that intend to use plants and microbes to enhance the extraction of economically valuable elements and contaminants from soil, and for biomass for bioenergy projects that intend to use microbes to enhance plant biomass production.

4. Materials and Methods

4.1. Plant Growth Experiment and Soil Amendment

The plant species used as test plants in this study were *Zea mays* cv *Badischer Gelber* and *Fagopyrum esculentum* cv *Moench*, which were grown under constant laboratory conditions of a temperature of 25 °C and light exposure time of 12 h per day. The plants were grown in 3 replicates, each in 2 kg of potted soils obtained from the vicinity of Technische Universität Bergakademie Freiberg, which represent typical soils of the Freiberg area of Germany [62]. Five seeds of each plant species were initially sown per pot but reduced to one plant per pot after 2 weeks post-germination. Plants grown in non-inoculated soil served as the reference for those grown in soils inoculated with *Bacillus amyloliquefaciens*. An inoculation rate of approximately 0.4% (0.4 mL of inoculum in 100 mL) per pot was used, and the soil was inoculated twice (100 mL of 0.4% inoculum mixture each time) within the 53-day growing period of the experiment, with a time interval of 2 weeks between inoculations. Rhizovital 42 (bioformulated *Bacillus amyloliquefaciens*), supplied by ABiTEP GmBH Berlin and containing 2.5×10^{10} CFU/mL (colony-forming units per milliliter) of *Bacillus amyloliquefaciens*, was the source of inoculum.

4.2. Sample Preparation and Analysis

4.2.1. Soil Samples (Before Inoculation)

According to Du Laing [63], readily available element fractions include the mobile/exchangeable and acid-soluble element pools. The concentrations of the elements in these fractions were determined via sequential extraction according to the methods described by Wiche and Heilmeier [6]. To determine the total element concentrations, 10 portions of the soil samples were dried at 105 °C and ground in a boron carbide mortar. Then, 0.5 g of the ground soil and 2 g of an equivalent mixture of Na_2CO_3 and K_2CO_3 were placed in a nickel crucible and thoroughly mixed for melting digestion, according to the methods by Alfassi and Wai [64]. The mixture was heated in a muffle furnace for 30 min at 900 °C, after which the samples were cooled and dissolved with 50 mL of a 2 M nitric acid and 0.5 M citric acid solution. The resulting solutions from the melting digestion and sequential extraction were diluted, and the concentrations of the elements were determined using ICP-MS (X series 2, Thermo Fisher Scientific, Dreieich, Germany). The accuracy of the analytical process was checked using certified reference material (NCS ZC73032 and NCS ZC73030) [65]. The results deviated by less than 10% from the certified values.

The physico-chemical properties of the uninoculated soil, the concentrations of the readily available soil element fractions, and the total element concentrations are reported in Table 4. Soil electrical conductivity was 32 µS/cm, while the soil organic matter content, determined by the loss of ignition, was 7.7 %. The soil pH was 6.2 and in the effective range for soil microbial functions and nutrient availability but not for the bioavailability of

most of the CRMs considered in this study [66,67]. The total concentrations of Ge and REEs were similar to those reported by Wiche et al. [62], with the total concentration of PTEs more than the threshold values allowed for European soils, as reported by Tóth et al. [68], which is due to previous mining activities in the region of Freiberg. Of the readily available PTEs, Pb had the highest concentration (36.6 μg/g), while the concentrations of readily available As, Cu, Co and Cr, and Co were 1.13 μg/g, 1.53 μg/g, 0.34 μg/g, and 0.34 μg/g, respectively. The readily available concentrations of the sum total of REEs (3.79 μg/g) were quite higher than that of Ge (0.02 μg/g). For the selected nutrients, the concentrations of the readily available fractions were P (58.9 μg/g), Fe (23.5 μg/g), Ca (2514 μg/g), and Si (117 μg/g). These concentrations mean that the soil was polluted but not nutrient deficient or infertile.

Table 4. Soil physico-chemical parameters and concentrations of elements.

4a: Soil Physico-Chemical Parameters	
Water content (*w*/*w*)	17.9%
pH value in aqueous solution	6.2
Conductivity	32.3 μS/cm
Organic matter content	7.7%
Nitrate concentration	147 mg/kg
Ammonium concentration	0.88 mg/kg
Phosphate concentration	136 mg/kg
Cation exchange capacity	9.1 cmol/kg

4b: Total Concentration and Concentration in Operationally Defined Fractions (μg/g) (mean ± SE)			
	Total concentration	Fraction 1	Fraction 2
Cu	175 ± 36	0.69 ± 0.04	0.84 ± 0.1
Pb	180 ± 41	5.6 ± 0.8	31 ± 3.2
Cr	111 ± 11	0.10 ± 0.02	0.23 ± 0.01
As	93 ± 25	0.39 ± 0.2	0.73 ± 0.2
Ge	1.84 ± 0.04	0.004 ± 0.001	0.014 ± 0.001
REET	157 ± 3.1	0.99 ± 0.1	2.80 ± 0.2
Ca	5875 ± 675	2282 ± 495	232 ± 45
P	1986 ± 89	33.3 ± 6.3	25.6 ± 8.3
Fe	29,337 ± 551	4.1 ± 0.4	19.4 ± 2.2
Co	24.3 ± 2.1	0.09 ± 0.01	0.24 ± 0.02
Si	141,455 ± 18,019	62.7 ± 9.6	54.7 ± 5.0

Fraction 1 = mobile/exchangeable element fraction, Fraction 2 = acid soluble element fraction. Values are means of 10 replicates except for P (total concentration), whose value is the mean of 7 replicates. Elements in bold letters have concentrations higher than permitted for European soils, as reported by Tóth et al. [66].

4.2.2. Plant Samples

During harvest, the plants were cut off at heights between 2–3 cm above ground level, weighed, and dried at 60 °C in an oven (model SIM 500, Memmert, Schwabach, Germany) for 48 h to obtain a constant weight. Subsequently, the dry mass of the samples was determined and pulverized to a fine powder using an ultra-centrifugal mill (model ZM1000, Retsch, Haan, Germany). Then, 100 mg of the dried pulverized plant samples were weighed out for digestion in a microwave (MLS-ETHOS plus, MLS GmbH, Dorsten, Germany) according to the methods by Krachler et al. [69]. Before digestion, the samples were mixed with 200 μL of ultra-pure water as well as with 1.9 mL nitric acid and left overnight to react before adding 600 μL of 4.9% hydrofluoric acid. After digestion, the samples were transferred into 15 mL centrifuge tubes, with volumes of up to 10 mL. For the measurement of trace elements, Ge, and REEs using ICP-MS (model X Series 2, Thermo Fisher Scientific, Dreieich, Germany), 1 mL each from the diluted samples were further transferred to 15 mL Teflon tubes before adding 100 μL of internal standards containing 1 mg/L of rhodium and rhenium, according to the methods by Krachler et al. [69], with volumes of up to 10 mL. The accuracy of the analytical process was checked using certified reference material

(NCS ZC73032 and NCS ZC73030) [62]. The results deviate by less than 10% from the certified values.

4.2.3. Soil DNA Extraction and Illumina Sequencing

Microbial DNA was extracted from approximately 250 mg soil, which had been collected immediately after plant harvest and preserved at −80 °C. The extraction procedure was done using a QIAGEN DNeasy Power Soil kit and based on the specifications of the manufacturer. Before storing the DNA extracts at −20 °C, the DNA concentrations in the extracts were examined with a NanoDrop ND-8000 spectrophotometer (Thermo Fischer Scientific, Dreieich, Germany). For the PCR, the DNA concentrations of the extracts were adjusted to 10–15 ng/µL. Amplification of the bacterial 16S rRNA gene V4 region was performed in triplicate for each sample with the universal primers 515f and 806r [70], which were equipped with Illumina adapter sequences. To ensure the correct amplification of the sequences, proofreading KAPA HiFi polymerase was used for all PCR reactions (KAPA Biosystems, Boston, MA, United States). The PCR reaction consisted of 7.5 µL of KAPA polymerase, 0.3 µL of each primer (10 µM), 5.9 µL of water, and 1 µL of DNA template, and was conducted with the PCR conditions summarized in Table 5 (PCR1). The PCR products were checked by gel electrophoresis, and triplicates for each sample were pooled together. After purification of the PCR products with the Agencourt AMPure XP kit (Beckmann Coulter, Krefeld, Germany), Illumina Nextera XT indices were attached to both ends of the bacterial fragments in a second PCR (PCR2, Table 5) in order to assign the sequences to the respective samples. The PCR products were purified using AMPure beads, and the DNA was quantified with the PicoGreen assay (Molecular Probes, Eugene, OR, United States). For an equimolar representation of each sample, defined volumes of the prepared bacterial amplicon libraries were pooled together. The fragment size and the quality of the final DNA sequencing library pool were again checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). Finally, paired-end sequencing of 2 × 300 bp was implemented on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) at the Department of Soil Ecology of the Helmholtz Centre for Environmental Research (UFZ, Halle/Saale, Germany).

Table 5. PCR conditions used for next-generation sequencing with Illumina for initial amplification of 16S rRNA gene region (PCR 1) as well as for the index PCR (PCR 2).

	Step	Temperature (°C)	Time (min:sec)
PCR 1			
25 cycles	Initial denaturation	95	**3:00**
	Denaturation	98	**0:20**
	Annealing	55	**0:15**
	Elongation	72	**0:15**
	Final extension	72	**5:00**
PCR 2			
8 cycles	Initial denaturation	95	**3:00**
	Denaturation	98	**0:30**
	Annealing	55	**0:30**
	Elongation	72	**0:30**
	Final extension	**72**	**5:00**

4.2.4. Bioinformatics Workflow

Demultiplexed sequences were processed using the "dadasnake" pipeline [71], which is based on the implementation of the DADA2 package [72] from the open-source program R (v. 3.6.1; R Core Team 2017) into Snakemake [73]. 16S rDNA amplicon reads were cut and filtered using the default settings of the pipeline. Read pairs were merged with a minimum overlap of 12 bp and zero mismatches, and chimeric reads were removed using

the consensus algorithm. For taxonomical classification of the 16S rDNA gene amplicon sequences, the Mothur implementation of the Bayesian Classifier (Schloss et al. [74]) and—as a follow up in the case of a missing classification—BLASTn were applied, referring to the SILVA database (version 132, non-redundant at 99%; [75]). The final output was comprised of an OTU table with the taxonomic classifications for all samples.

4.2.5. Statistical Analysis

The statistical differences between the treatments for each plant species for shoot contents (amount accumulated), element concentrations, and shoot yield were evaluated using Welch's analysis of variance (ANOVA) at a significance level of $p < 0.1$ using IBM SPSS Statistics 26 software. Significant differences ($p \leq 0.1$) between the means indicated are indicated by an asterisk * in the figures. The bar plots and PCoA were created with R, version 4.0.5, using the "vegan" and "ggplot2" packages.

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Manuscript I

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Relationships between essential and non-essential elements in plants with different nutritional strategies and silicon absorption capacities

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Abstract

We explored relationships between essential and non-essential elements (aluminum (Al), cadmium (Cd), rare earth elements (REE)) in plants with different nutrition strategies, and the influence of silicon (Si) on these interactions. *Brassica napus, Cucumis sativus*, *Lupinus albus*, *Pisum sativum* and *Zea mays* were cultivated in a semi-hydroponic and treated with Al, REE (La, Ce, Nd, Gd and Er), Al+REE, and Al+Cd+REE at 10 and 100 µmol L^{-1}. Plants treated with Al+Cd+REE received 1.5 mM silicon (Si) or no Si. After harvest shoot elemental composition was analyzed by ICP-MS. *Lupinus albus* and *Z. mays* tolerated high treatment concentrations, while *B. napus*, *C. sativus* and *P. sativum* responded with declining shoot growth. Aluminium increased REE accumulation in the plants. Without adding Si, *B. napus* accumulated higher Cd and REE than other species. Supplying 10µM Al+Cd+REE with Si decreased Cd and REE accumulation in all plant species. Conversely, at 100 µmol L^{-1}, adding Si increased Cd and REE accumulation in the Si-accumulators (*Z. mays*, *C. sativus*), but no effect on the non-Si-accumulators (*B. napus*, *L. albus*). The synergetic effects between Al and REE might explain the co-hyperaccumulation phenomena reported for REE-hyperaccumulators. Utilizing Si-fertilizers could be a promising approach in phytoremediation to enhance phytoextraction efficiency.

Keywords: silicon absorption, interactions, resistance, tolerance, rare earth elements, aluminum, cadmium

Introduction

Among the spectrum of phytoavailable elements in the soil or other growth media, plants are exposed to not only the 14 essential nutrients but also a large spectrum of non-essential elements such as aluminum, silicon, cadmium, and rare earth elements (Al, Si, Cd, REE). As for any other essential trace elements, the toxicity of the non-essential elements depends on the plant-available concentration in the growth media, plant species, the chemical speciation of the elements, and the overall nutritional status of the plant (Barker and Pilbeam, 2015). Excessive concentrations of trace elements may interfere with the uptake of essential elements, disrupt membrane integrity and plant metabolic activity, interfere with nutrient homeostasis (Hakeem et al., 2015) , and consequently cause oxidative stress (Schutzendubel, 2002; Sharma and Dietz, 2009; Rascio and Navari-Izzo, 2011; Viehweger, 2014) . Especially in acidic soils where cation-solubility is high, plants may suffer from an excess of essential elements of which Mn-toxicity has been most profoundly studied (Faria et al., 2021). At the same time, the roots are exposed to high concentrations of non-essential cations, especially Al, Cd and REE in the soil solution), whose solubility increases with decreasing pH, which leads to multi-element stress (Marschner, 1995; White, 2012).

Similarly, when plants suffer a nutrient deficiency, the related functional root traits targeting solubilization of essential nutrients in soils, especially rhizosphere acidification and release of carboxylates, are generally not element specific and mobilize a variety of non-essential, potentially toxic elements as it has been described for Al, Cd, Pb and REE (Wiche et al., 2015; Monei et al., 2022). While element excess in highly mineralized soils has been most profoundly studied for a variety of potentially toxic non-essential trace elements, the interactions between elements under nutrient limitation are rarely studied. It is reasonable that this spectrum of non-essential elements brought into soil solution additionally impacts plant availability of essential elements and physiology depending on the type of elements, concentration and chemical speciation and plant species. Moreover, most studies dealt with stress from single elements without considering their interactions. It is generally assumed that plants can adapt to toxicity stress by avoidance or tolerance mechanisms (Wang et al., 2017; Nikalje and Suprasanna, 2018). In avoiding or tolerating toxic elements, plants prevent toxic metal ions from entering the cellular cytoplasm, or they rapidly translocate the toxic elements to the shoot, where they are stored in non/less-metabolically active plant compartments or detoxified by changes in their chemical forms. In this manner, plants confer Al resistance by excluding the ion from the root symplast by elevated secretion of organic metabolites and forming carboxylate complexes (Lambers et al., 2013; Garau et al., 2021). In other instances, plants tolerate the Al within the symplast to resist any harm that may be triggered by elevated Al^{3+} concentrations (Brunner and Sperisen, 2013; Nikalje and Suprasanna, 2018; Wang et al., 2020). Recent studies demonstrated that not at least some non-essential elements, though they do not possess a functional role in plant metabolism, may support plant growth under various conditions of stress, including the deprivation or excess of the essential cognates. Low concentrations of Al and Cd and REE may support plant growth under various conditions of stress, especially in plants with high Al or Cd tolerance, and may furthermore increase nutrient acquisition (Rascio and Navari-Izzo, 2011; Zia-ur-Rehman et al., 2015; Bojórquez-Quintal et al., 2017; Alejandro et al., 2020). However, high concentrations of these elements may counteract the benefits listed (Liu et al., 2021).

Among the beneficial elements, Si has been best described regarding its beneficial effects on plants which are particularly interesting because of its high abundance in the earth's crust that plant roots are inevitably exposed to, which may, in turn, be beneficial in processes such as phytomining (Guntzer et al., 2012; Adrees et al., 2015; Bhat et al., 2019; Pavlovic et al., 2021). Ma (2004) first evidenced the interactions between soil silicon and essential elements, and that the availability of silicon increases the availability of phosphate and vice versa. Lambers (2022) recently showed that carboxylates released during phosphorus and micronutrient acquisition influence Si availability, which suggests a functional role of silicon not only in Si accumulating grasses but also in forbs.

The form of Si readily available to plants for uptake is monosilicic acid (H_4SiO_4) (Marschner, 1995; Epstein, 2001; Hayes et al., 2014; Pavlovic et al., 2021) which is taken up by Lsi aquaporins that are mainly present in grasses that, described to be Si accumulators showing up to 10% Si of the dry weight (Epstein, 2001; Ma and Yamaji, 2006; Pontigo et al., 2015). In contrast, most dicots lack the Lsi transporter; thus, their Si concentrations rarely exceed 0.1%. However, some dicot species, such as cucumber undergo silicification (Guntzer et al., 2012). In most plants, the uptake of Si can either be passive (cucumber, soybean and strawberry) or active (rice), whereas other species may exclude Si from being taken up (other legumes and tomato) (Ma et al., 2001a; Liang et al., 2005; Chen et al., 2018; Yan et al., 2018). Thus, in monocots, the beneficial effects of Si mostly derive from tolerance mechanisms at cellular or tissue level, while in non-accumulating dicots, Si may interfere with processes at the soil–root interface through stress avoidance. Besides the activation of reactive oxygen species (ROS) detoxification, silicon inhibits heavy metals absorption and transport in the root system in the avoidance mechanism, further increasing cell wall adsorption (Farooq et al., 2013). The formation of cell barriers may lead to Si-Cd co-precipitation (Liang et al., 2007). Wang et al. (2004) indicated that in alleviating Al-

toxicity with Si, 85% of Al was bound to the apoplast in the root cell wall. Silicon can also increase metal tolerance to elements such as Al, Cd and Mn (Da Cunha and do Nascimento, 2009; Ma, 2010; Shen et al., 2014; Adrees et al., 2015). The formation of non-toxic silicate complexes in the soil solution has been demonstrated for Al that forms Al-Si complexes such as hydroxyl-aluminosilicates (HAS) which may also bind to REE (Ma et al., 2016; Bhat et al., 2019; Hodson and Evans, 2020).

Greger et al. (2018) and Pavlovic et al. (2021) demonstrated that applying Si treatment increased the uptake of the mineral nutrients Ca, Mn, and Fe in maize, pea and wheat. Nevertheless, little is known about the interaction between Si and essential and non-essential elements and the role of REE in Si-mediated mechanisms in non-Si accumulators. To reveal these interactions, five plants species with different nutritional strategies where subjected to a variation of different combinations and levels of REE, Al, and Cd and Si in a greenhouse experiment. The species were *Zea mays* and *Cucumis Sativus* (both prevalently known to be Si-accumulators), *Brassica napus* (a heavy element accumulator), *Lupinus albus* (an excluder plant) and *Pisum sativum* (an excluder). For these species, we explored the relationship between Si accumulation and uptake of essential nutrients and non-essential elements Al, Cd, and REE This interplay between species-specific physiology and nutritional status concerning the availability and function of essential and non-essential, beneficial elements has been broadly neglected hitherto. However, its understanding would improve our general understanding of the adaptations of plants to their natural multi-elemental growth environment.

Materials and Methods

Plant growth and treatment

Five species with differing nutrient strategies, rapeseed (*Brassica napus* L. cv Genie), white lupine (*Lupinus albus* L. cv Feodora), pea (*Pisum sativum* L. cv Karina), maize (*Zea mays* L. cv. Badischer Gelber) and cucumber (*Cucumis sativus* L. cv Paksa) were cultivated in a greenhouse set up. The plants were grown and cultivated in 2 L pots. In preparation for the cultivation, the bottoms of the pots were layered with pebbles and filled with 200 g of a peat-based semi-hydroponic substrate. Four seeds of each plant species were germinated per pot with 5 replications for each species and after culling, one plant per pot was left to grow. After the first week of cultivation, the plants were weekly supplied with 200 mL of a 1:5 strength Hoagland solution prepared according to Hoagland & Arnon (1950) . Two weeks following cultivation, different treatments were applied. One group served as reference plants, which only received the Hoagland solution, and the other plants were treated with five different solutions namely: aluminum (Al), rare earth elements (La, Nd, Ce, Gd and Er, further denoted REE), Al+REE, the mixture of Al+Cd+REE (Trace elements, further denoted TE) at concentrations of 10 µmol L^{-1} and 100 µmol L^{-1}, respectively, for each element. To determine the effects of Si the treatment TE was complimented with another treatment, TE + Si, composed of TE and 1.5 mM Si. *Pisum sativum* was treated with Al and REE only, and *C. sativus* with Al+REE+Cd with Si and without. In preparation for the treatment solutions, Al was added as $Al_2(SO_4)_3$, Cd as $Cd_2SO_4 \cdot H_2O$, rare earth elements consisted of La, Nd, Ce, Gd and Er (prepared from $REE(NO_3)_3$ (Merck) and Si as Na_2SiO_3 (Sigma-Aldrich Chemie GmbH). The plants were incubated in a semi-controlled growth chamber in a fully randomized design. The average temperature was 22.4 °C min: 10.8 °C, max: 45.3 °C), active photosynthetic photon flux density was 600 µmol s^{-1} m^{-2}, and the average relative humidity was measured at 61.2 %. The total duration of the experiment was 45 days (~6 weeks) until the plants were harvested. The shoots were cut 2 cm above the growth substrate surface when harvesting the plants and subsequently processed for trace element analysis.

Characterization of the Substrate

The experiment was conducted using a semi-hydroponic substrate, a peat-based growing media from Klasmann-Dreilmann (Geeste, Germany). The substrate was selected because of its low available Si, REE, and Cd concentrations, enabling control of element availability on a solid growth substrate. The initial mineral composition in the substrate, nitrate (NO_3^-), phosphate (PO_4^{3-}) and ammonium (NH_4^+) was measured after extraction with deionized water, calcium lactate/acetate and potassium chloride using spectrophotometry, following the method in Wiche et al. (2016) and Monei et al. (2022). The plant available nutrient concentrations were 30 mg kg^{-1} PO_4^{3-}, 13 mg kg^{-1} NH_4^+ and 192 mg kg^{-1} NO_3^-. Cation exchange capacity was determined following Wiche et al. (2017), and it was 105 cmol kg^{-1}. The pH (H_2O) of the growth substrate was 6.5; and electrical conductivity 616 µS cm^{-1} m.

A sequential extraction was conducted according to Wiche et al. (2017) and Monei et al. (2022) to determine the distribution of the elements in the substrate. The extraction process follows six fractional steps undertaken in sequence, including exchangeable elements (F1), acid-soluble elements (F2), elements in the oxidizable matter (F3), amorphous oxides (F4), and crystalline oxides (F5) (Wiche et al., 2017; Monei et al., 2022). Each step was followed by a

collection of supernatants from the centrifuged sample and later analysed by ICP-MS to determine the concentration of Ca, P, Mn, Fe, Al, Cd, Si and REE (La, Nd, Ce, Gd and Er) represented in Table 1. The REE were divided into light rare earth elements (LREE- represented by La, Nd and Ce) and heavy rare earth elements (HREE- represented by Er and Gd).

Assuming that Fractions F1 and F2 represent the easily plant available element fractions, the substrate contained high concentrations of mobile, exchangeable, and acid soluble Ca (4897 mg kg^{-1}) and P (113 mg kg^{-1}), which are comparable to moderately fertile soils (Wiche et al., 2017; Monei et al., 2022). In contrast, substrate concentrations of available trace nutrients (Fe, Mn), and non-essential elements (Si, Al, Cd, LREE, and HREE) was two orders of magnitude lower compared to those in soils (Wiche et al., 2017; Monei et al., 2022).

Analysis of Harvested Plant Material

The harvested plants were collected and dried in an oven for up to 48 hours at 60 °C. The harvested plants were weighed before and after drying and subsequently ground to a fine powder in preparation for analysis (Schwabe et al., 2021; Monei et al., 2022). A 100 mg subsample of the ground powder was transferred into Teflon digestion tubes. For microwave digestion (Ethos plus 2, MLS), the preparation method by Krachler (2002) was followed. The method entails the addition of 1.9 mL nitric acid 65 % (HNO_3^-) and 0.6 mL hydrofluoric acid 4.8% (HF), which was preceded by moistening the subsample with 0.2 mL deionized water. The digested samples were further analysed through ICP-MS (X-SERIES 2, Thermo Scientific) to measure the concentration of Ca, P, Mn, Fe, Al, Cd, Si, Gd, Er, La, Nd, Ce, and Gd using 10 µmol L^{-1} rhodium and rhenium internal standards (Wiche et al., 2017; Monei et al., 2022).

Statistical Analysis

All the data of the shoot biomass and the concentrations of Ca, P, Fe, Mn, Si, Al, Cd and REE were processed using an analysis of variance (ANOVA). Bartlett's test was used to verify the homogeneity of variances preceding the analysis. If a violation of the ANOVA assumptions occurred, the data were log-transformed. Welch's ANOVA (α = 5%) was considered when the assumptions were still violated. Fishers LSD posthoc test (LSD-test) was used to indicate significant effects between the different treatments and the reference plant and between low and high concentrations of the treatments. All statistical calculations were conducted using IBM SPSS Statistics 25.

Results

Effects of Al and REE on biomass production and shoot nutrient concentrations

The biomass produced from *B. napus*, *L. albus*, *P. sativum*, and *Z. mays* differed according to the treatment regime (Table 2). In *B. napus* and *Z. mays,* both Al treatments tended to decrease the shoot biomass, but this effect was not statistically significant at α = 5%. In *L. albus,* no changes in shoot biomass could be observed, regardless of the concentration of Al given with the nutrient solutions. As an exception, the biomass of *P. sativum* showed an increased growth response following the Al-treatments when compared to the reference plants. The application of REE at 10 µmol L^{-1} had no significant effect on *B. napus*. *Pisum sativum* showed a significant increase in biomass by 53% and 84%, when the plants received 10 and 100 µmol L^{-1} REE, respectively. In contrast, for both *L. albus* (28%) and *Z. mays* (32%), the biomass reduced, especially at low REE concentrations (Table 2). The mixture of Al+REE did not influence plant growth of *L. albus* and *Z. mays*, irrespective of the concentrations. However, in *B. napus,* both concentrations of the element mixture substantially reduced growth up to 2.8-fold.

Considering the shoot nutrient concentrations, the addition of low doses of Al to *B. napus*, *L. albus* and *Z. mays* increased shoot Si and Ca concentrations with the most strongly pronounced effect on Si in *Z. mays* (Table 2). In contrast, *P. sativum* did not respond with altered Si concentrations and showed decreasing Ca concentrations. Low doses of Al led to increased concentrations of P, Fe, and Mn in *L. albus* and *P. sativum*, but did not influence P, Fe and Mn in *B. napus*. Additionally, *Z. mays* responded with significantly increased P and Fe concentrations, while Mn concentrations remained unchanged. High Al-doses did not impact Si accumulation in the plants except in *Z. mays* which showed significantly higher Si concentrations in Al-treated plants compared to the reference. Similar to responses to low Al-doses, 100 µmol L^{-1} Al increased Ca in *B. napus* and *L. albus*, while Ca remained constant in *P. sativum* and decreased significantly in *Z. mays*. Moreover, high Al- doses significantly increased P concentrations in all investigated species but did not change Fe and Mn, except in *L. albus*, which responded by substantially elevated Fe and Mn concentrations.

The addition of low doses of REE did not change Si, Fe and Mn in *B. napus* but led to an increase of Ca and P concentrations in the shoot biomass. Also, in *L. albus* Si concentrations were not affected by REE but Ca, P, Fe and Mn concentrations increased substantially by 118%, 74%, 118%, 111%. This strong increase in phosphorus and micronutrients following supply with low-dosed REE was also observable in *P. sativum* and *Z. mays* and REE treated plants of *Z. mays* were additionally characterized by 117% higher Si concentrations compared to the reference plants. Generally, there was no difference in the response of *B. napus*, *L. albus* and *P. sativum* to the different concentrations of REE. Finally, when the

plants received both Al and REE together (Al+REE), the low doses increased Ca, P, and Fe in *B. napus* and *L. albus* (including Mn), and only Si, P and Mn in *Z. mays*. When the mixture was applied in high doses, *B. napus*, *L. albus* and *Z. mays* showed the same pattern for the reported macro- and micronutrients, but *B. napus* and *L. albus* were additionally characterized by elevated Si (*B. napus*: 75%, *L. albus*: 56%) concentrations compared to the reference plants.

Effects of Al and REE on the concentrations of Al and REE in the different plant species

Table 3 summarizes the uptake of Al, LREE and HREE for the different species with regards to the treatments. From all plant species tested, concentrations of Al, LREE and HREE were the highest in *B. napus* irrespective of the treatment. Reference plants of *L. albus* showed the lowest concentrations of Al and REE. The addition10 µmol L^{-1} Al did not significantly affect shoot Al concentrations in all investigated plant species except in *L. albus*, where Al concentrations increased by 126% compared to the reference. Surprisingly, all plant species showed a substantial increase in REE concentrations, especially LREE, when treated with 10 µmol L^{-1} Al and this effect was the strongest in *L. albus* (3680% increase) and decreased in the order *L. albus* > *P. sativum* (438% increase) > *B. napus* (167% increase) > *Z. mays* (263% increase). When Al was supplied at 100 µmol L^{-1}, Al concentrations increased in all species showing a strong response in *L. albus*, *B. napus* and *P. sativum* (166%, 70% and 139% increase) and lower effects in *Z. mays* (59% increase). High Al-doses did not alter REE concentrations in *B. napus* and *Z. mays*, whereas *P. sativum* strongly responded with an increase of LREE and HREE concentrations (950% and 1550%, respectively).

When REE was added to the plants, LREE and HREE concentrations in all plant species steadily increased with increasing REE supply. At 10 µmol L^{-1} REE, Al concentrations increased significantly, showing the strongest response in *B. napus*. In *B. napus* and *L. albus* adding high REE led to an increase in Al concentration when compared to the reference plants. In contrast, 100 µmol L^{-1} REE did not significantly influence Al in *P. sativum* and *Z. mays*. Finally, high Al+REE supply, only *L. albus* responded with increased Al. The concentrations of REE steadily increased with increasing Al+REE-supply compared to the reference, but the REE concentrations remained lower than when treated with 10 µmol L^{-1} REE.

Shoot element absorption in different plant species responding to Al and REE supply

Figure 1 shows the uptake of micro and macro nutrients accumulated in the tested species, and Figure 2 Al, LREE and HREE. The reference plants of B. *napus*, *P. sativum*, *L. albus*, and *Z. mays* have different micro and macronutrient uptake. *B. napus* had a higher uptake of calcium and phosphorus, like *Z. mays*. Furthermore, *Z. mays* had higher Fe uptake than *P. sativum* and *L. albus* and the highest Mn than the other species. *Brassica napus* and *Z. mays* had similar uptake of Si, Al, LREE, and HREE. But *Z. mays* had higher uptake of Si, Al, and HREE compared to *L. albu*s and *P. sativum*. Also, *B. napus* had a high uptake of LREE compared to *L. albus* and *P. sativum*.

Treating the plants with 10 µmol L^{-1} Al led to increased Al uptake in *L. albus* and *P. sativum*, but no change in *B. napus* and *Z. mays*. Low Al doses decreased Mn and Si uptake in *B. napus* and *Z. mays*, and increased Ca, P, Mn, and Fe in *L. albus* and P. *sativum*, but decreased Si uptake in both. The uptake of LREE and HREE in *B. napus* remained unchanged but increased in the other tested species, Figure 2. Al treatment at 100 µmol L^{-1} increased Al uptake in *L. albus* and *P. sativum* but had no effect in *B. napus* and *Z. mays*. High Al doses led to decreased Ca and increased Si uptake in *Z. mays*. Furthermore, Ca, P, Fe, Mn, and Si uptake increased in *P. sativum* and *L. albus* at high Al doses but did not change in *B. napus*. The uptake of LREE and HREE in *B. napus* and *Z. mays* remained unchanged but increased in *P. sativum* (2278% and 3500%) and in *L. albus* (180% LREE and no effect in HREE), Figure 2.

Low doses of REE increased REE uptake in all species, with *B. napus* having the highest uptake, indicating higher LREE than HREE uptake in all species (Figure 2). Low REE doses increased uptake of Ca, P, Fe, and Si in *B. napus*, Fe in *L. albus*, P and Fe in *P. sativum*, but decreased Ca and increased Si in *Z. mays*. Low REE also increased Al uptake in *B. napus*, *L. albus* and P. sativum, except in *Z. mays*. Increasing the REE treatment (100 µmol L^{-1}) led to increased LREE and HREE in all species (Figure 2). The high doses of REE had no effect on the uptake of Ca, P, Mn, and Si, as well as showing a decrease in Fe (34%) in *B. napus* and *Z. mays*, further showing increased Si (125%) in *Z. mays*. However, these high REE doses proved to increase Ca (only in *L. albus*), P, Fe, Mn in *L. albus* and *P. sativum*. Furthermore, Si decreased in *L. albus* but increased in *P. sativum*.

In combining Al and REE (Al+ REE) at low doses, there was no effect on Al uptake in *Z. mays,* but a 45% decrease in *B. napus* and an increase in *L. albus* (Figure 2). Furthermore, REE uptake increased in all species compared to the reference plants, with the highest increase in *L. albus*. The uptake of Ca, P, Fe, Mn, and Si significantly decreased in *B. napus*; however significantly increased in *L. albus* and had no effect on Si whereas *Z. mays* showed no change in Ca, Fe, and Mn uptake but increased in P and Si, (Figure 1).

At 100 µmol L^{-1} Al+REE all the species indicated an increased LREE and HREE uptake, which differed significantly from the low doses (Figure 2). The uptake of Al only decreased at high Al+REE doses in *B. napus*. *Lupinus albus* had the highest increase in REE at high doses (2900% LREE and 8150% HREE), and in *B. napus*, the addition of high Al+REE doses

resulted in decreased Ca, P, Fe and Si. Whereas in *L. albus*, the uptake of Ca and P increased (93% and 33%); in *Z. mays*, the uptake of Ca decreased (46%) and Si increased (78%).

The effect of Si application on biomass production and element concentrations

The application of TE solution consisting of Al, Cd and REE without Si did not affect shoot growth in *B. napus* and *L. albus* at any of the concentrations supplied (Table 4). In *C. sativus,* both concentration levels of TE strongly reduced shoot biomass by 48% and 49%, but there was no difference between the concentrations regarding the effect strength. Moreover, in *Z. mays,* low TE doses did not affect plant growth, while high concentrations reduced shoot biomass by 40%.

When it comes to the accumulation of rare earth elements, *B. napus*, *C. sativus* and *L. albus,* and *Z. mays* indicated a higher accumulation of LREE than HREE, despite the treatment type. Treating *B. napus*, *C. sativus*, *L. albus* and *Z. mays* with the 10 µM TE treatment resulted in a higher accumulation of both LREE (834%, 581%, 1364%, and 966% respectively) and HREE (2785%, 1188%, 3399% and 2007%, respectively) than the reference plants. Similarly, Cd concentrations in all species raised steadily with increasing element supply from 10 µmol L^{-1} to 100 µmol L^{-1} and this effect was the most strongly pronounced in *B. napus*, while *L. albus* responded weakly to the elevated element concentrations in the growth medium. The application of TE did not influence shoot Al concentrations in the tested plant species except in *C. sativus,* where high Al-doses in a mixture with Al, Cd and REE increased Al by 72% (Table 5).

The application of TE at low doses significantly increased Si, Ca, and P concentrations in *B. napus* and Ca, P, Fe and Mn in *L. albus*. Also, in *C. sativus,* Ca significantly increased by 561%, while Fe significantly decreased by 43%. In *Z. mays*, low TE concentrations solely influenced Ca, showing a 200% increase, and all other elements remained unaffected, as seen in Table 4. When the plants of *B. napus* received high element concentrations, Ca concentrations of the treated plants were far higher than the reference, but there was no difference between the low and high TE treatment. Also, in *B. napus,* all other elements, including P and Fe, remained unchanged. In *C. sativus,* the treatment had no influence on Si and increased Ca, P and Mn in *L. albus* and P and Si in *Z. mays* though the silicon concentration in the nutrient solution was not altered.

Adding Si to the plants did not significantly increase shoot Si concentrations in *L. albus* but significantly increased shoot Si in *C. sativus* and *Z. mays* by up to 316% and 416%, respectively. The addition of Si together with the TEs significantly reduced the shoot biomass in *B. napus* compared to the reference, but there was no difference between the TE with Si and the TE without Si. Irrespective of the TE concentrations, plants of *C. sativus* treated with Si still showed significantly lower biomass than the reference, but the biomass tended to increase in the presence of Si compared to TE-treated plants without Si (an increase of biomass by 34% and 20%, respectively). In contrast, similar to *B. napus*, in *Z. mays,* the shoot biomass decreased in the presence of Si, showing significantly lower biomass compared to the reference, especially when the plants were exposed to low elements in the TE (Table 4).

The addition of Si to plants exposed to low concentrations of Al, Cd and REE (TE+Si) significantly increased the concentrations of the essential nutrients P and Si in *B. napus* (Table 5). In *C. sativus* TE+Si significantly decreased the concentrations of Cd, LREE and HREE by 52%, 58%, 43%. However, TE+Si led to decreased Cd and REE concentrations in all species except *L. albus* which showed reduced Cd concentration but had similar LREE and HREE concentrations compared to plants without Si treatment. When TE were amended at 100 µmol L^{-1}, adding Si strongly decreased Ca and increased Fe in *B. napus* and *C. sativus* by 115% and 60%. Similarly, it increased Fe and Mn in *L. albus* and *Z. mays*, while Ca and P concentrations were not affected in these species compared to the low doses. Simultaneously, the addition of Si together with high concentrations of TE elements significantly increased the concentrations of Al in *B. napus*, increased Al and REE concentrations in *C. sativus* and increased Cd and REE in *Z. mays.*

Shoot absorption of essential and non-essential elements as affected by silicon

When the treatment TE (AL + Cd + REE) was applied at 10 µmol L^{-1}, there was no influence on the uptake of Al in *B. napus*, *Z. mays*, and *C. sativus* but increased it in *L. albus* (96%) compared to the reference plants. On the other hand, the uptake of Cd and REE increased significantly in all species, with *Z. mays* showing the highest Cd compared to *B. napus*, *C. sativus*, *L. albus*, (Figure 4). Adding the low TE dose indicated no influence on Al in *B. napus* and *L. albus* but a decrease in *C. sativus* (49%) and *Z. mays* (35%) compared to the reference plants. Calcium uptake increased in all tested species. However, the effects on Si, P, and Fe uptake vary by plant species, with Si increasing in *Z. mays* and decreasing in *C. sativus*, and P and Fe decreasing in *C. sativus*. Mn uptake also varied, with decreases in *B. napus* and *C. sativus* and increases in *L. albus*.

At high TE (100 µmol L^{-1}), Cd and REE uptake increased in all the species, with the highest uptake of Cd and LREE observed in *B. napus*. Additionally, there was a 2-fold increase in LREE and HREE in *Z. mays* and a 3-fold increase in *C. sativus*. However, in *C. sativus*, Al decreased by 49%. The added high doses of Al, Cd and REE mix showed no effect towards Al in *B. napus* and *L. albus*. Interestingly, increasing the TE dose led to a 76% decrease in Ca uptake in *Z. mays*. The uptake

of P was also affected, with 46% decrease in *B. napus*, 57% decrease in *C. sativus*, and no change in *L. albus*. Silicon uptake increased by 2475% in *Z. mays*. Furthermore, Mn uptake varied across the species, which increased in *L. albus* (105%) and decreased in *C. sativus* (57%).

The introduction of Si to TE treatment increased the uptake of Cd in all species, Figure 4. The uptake of REE also increased with high doses of TE+Si in *C. sativus* and *B. napus*. The uptake of REE also increased significantly when high doses of TE+Si were applied, with *C. sativus* having the highest LREE (67 µg) and *B. napus* the highest HREE (37 µg). The highest LREE and HREE were seen in all tested species when treated with high TE+Si concentrations compared to their reference plants and TE. Uptake of Al increased 2-fold in *B. napus* and *Z. mays* with low Si concentrations, unlike TE 10 µmol L^{-1}, which led to no differences. There was no significant difference between 100 µmol L^{-1} TE and TE+Si in terms of Al uptake. The uptake of Si in *B. napus* was not affected by Si supply.

When *C. sativus* was treated with 10 µmol L^{-1} TE+Si, P uptake decreased by 2.9-fold in comparison to the reference plants (Figure 3). Similarly, increasing the concentration of TE+Si did not differ from 10 µmol L^{-1}. The uptake of Ca in all the species followed the decreasing order *B. napus* > *C. sativus* > *Z. mays* > *L. albus* when the plants were treated with TE+Si 10 µmol L^{-1}. Compared to the reference plants, Ca uptake in *B. napus*, *C. sativus*, and *Z. mays* increased significantly when the plants were treated with TE+Si at 10 µmol L^{-1} (203%, 326% and 75%, respectively). However, increasing TE+Si concentration led to a decrease of Ca in *B. napus* and in *Z. mays*. In *C. sativus*, 100 µmol L^{-1} TE+Si led to decrease in Ca uptake, which was 23% higher than 10 µmol L^{-1} TE+Si. Unlike in the other species, the application of 100 µmol L^{-1} TE+Si led to decreased Ca uptake in *L. albus* compared to the reference plants.

The application of TE+Si increased Cd uptake in *C. sativus* by up to 7.8-fold. The uptake of LREE and HREE were higher in all species with TE+Si treatment compared to the reference plants, and there was no difference between TE+Si and TE in all species, Figure 4. However, increasing TE+Si concentration led to an increase in LREE and HREE. High doses of TE+Si had no influence on Si uptake in *B. napus*, but significantly increased in C*. sativus* but decreased in *L. albus*, Figure 3. At 100 µmol L^{-1} TE+Si decreased P in *B. napus*, and *in L. albus* it led to decreased Ca, P, Fe, and Mn. In *C. sativus*, the presence of high TE+Si had no influence on Ca, P, and Fe, but led to increased Mn. In *Z. mays*, the uptake of Ca, P, and Fe were not influenced by high TE+Si but led to increased Mn.

Unlike how there was no effect at the application of TE in *L. albus*, in *C. sativus*, TE+Si increased Cd uptake by up to 7.8-fold (Figure 4). The uptake of LREE and HREE indicate the same pattern as with the low treatment dose, where also LREE > HREE (Figure 4). The application of TE+Si 10 led to an increase in LREE (277%, 899%, 452%, 207%) and HREE (1100%, 1975%, 869%, 450%,) in *B. napus*, *L. albus*, *C. sativus* and *Z. mays*, respectively compared to the reference plants, and there was no difference between TE+Si and TE in all species. However, increasing TE+Si 100 µmol L^{-1} concentration led to a 17-fold increase in LREE and HREE compared to TE+Si 10 µmol L^{-1}. High doses of TE with added Si had no influence on Si uptake in *B. napus*. Treating *C. sativus* with 100 µmol L^{-1} TE+Si led to the uptake of Si significantly higher than the TE treatment (260%) as seen in Figure 3. In *L. albus*, Si uptake minimally decreased in the presence of high TE+Si. In *L. albus*, high doses differed by 108% from TE and 207% with low TE+Si. The 100 µmol L^{-1} TE+Si treatment only decreased P in *B. napus,* in *L. albus*, Ca, P, Fe and Mn (Figure 3). In *C. sativus*, the presence of the high dose of TE+ Si had no influence on Ca; furthermore, there was decrease in P and Fe, with no difference between the high and low doses for all these elements. Lastly, in *Z. mays,* the uptake of Ca, P, and Fe were not influenced by the high concentration of TE+Si but led to increased Mn (44%) compared to the reference plants, which did not differ from the low TE+Si.

Discussion

The influence of Al and REE on plant growth and shoot mineral composition

The literature indicates that Al and REE exhibit a similar behaviour in the soil plant system, which has been attributed to the chemical similarities of the trivalent cations during element acquisition in the rhizosphere and plant uptake (Pletnev and Zernov, 2002; Fehlauer et al., 2022; Liu et al., 2022). Aluminium and REE have been most profoundly studied regarding the detrimental effects on plant growth and metabolism. Exposure of plants to high concentrations of Al primarily leads to suppression of root growth, and induction of nutrient deficiency, especially of Ca, when plants are exposed to high concentrations (Jian et al., 1998; Ma et al., 2001b; Kochian et al., 2004). Rare earth elements share chemical similarities to Ca in terms of their ionic radii and thus have been successfully used in physiological studies as Ca channel blockers to trace Ca metabolism (Grosjean et al., 2019; Ascenzi et al., 2020). However, it has been demonstrated that low concentrations of Al and REE (µM range) can promote plant growth through enhanced photosynthesis, enzyme activities and improved plant nutrition (Tyler, 2004; Bojórquez-Quintal et al., 2017).

In our experiment we focused on the mineral composition and development of plant shoots and did not consider root growth and element accumulation in roots. Thus, our experiment primarily explores how changes in exposure of

plants species to different doses of Al and REE impact shoot development and mineral composition and does not allow the elucidation of the processes involved. The peat material used had a high cation absorption capacity. Hence, compared to hydroponic studies, element accumulation was influenced by the species-specific capacity element uptake and acquiring elements from the sorbed element pools of the peat (Table 1). Indeed, the reference plants watered with Hoagland's solution exhibited high variability in nutrient concentrations (Table 2) with comparable low Ca, P, Fe and Mn concentrations in *L. albus* and the highest concentrations in *B. napus* (Table 2). All plants contained Ca, P, Fe and Mn above the critical level of 5 mg g^{-1}, 2 mg g^{-1}, 100 µg g^{-1} and 50 µg g^{-1} (Marschner, 1995), except *P. sativum* that was deficient in Fe and Mn (Table 2), most probably due to the very limited capacity of this species to respond to nutrient deficiency by changes in rhizosphere chemistry (Pearse et al., 2007).

Zea mays and *L. albus* showed no biomass changes following Al and REE treatment, underlining the species capacity to cope with high metal concentrations in the soil solution (Tolrà et al., 2009). *Lupinus albus* responded with increased Al concentration but the concentrations in shoots were largely independent of the concentrations in the watering solutions, while in *Z. mays* there were no changes in Al concentrations and net shoot uptake. For *Z. mays* in particular, this suggests an element exclusion during the stage of ion uptake and/or preferential accumulation and detoxification of Al and REE in the roots (Giannakoula et al., 2008; Ding et al., 2022), whereas in *L. albus* element exclusion might be accompanied by an effective detoxification on cellular/tissue level, most probably through carboxylates (Anoop et al., 2003; Peñaloza et al., 2004; Valentinuzzi et al., 2016; Quiñones et al., 2021).

Unlike *L. albus, B. napus* responded with lower shoot biomass following a supply with different levels of Al and REE, except when supplied with low doses of REE. Concomitantly, *Brassica napus* did not show changes in shoot Al concentrations and contents but responded the strongest regarding the uptake and accumulation of REE (Table 3, Figure 2). For *B. napus* this may indicate a higher toxicity of Al than REE and the Al might have reduced root growth and thus Al uptake by the plants (Kidd and Proctor, 2000). Similar to the results obtained by Kidd and Proctor (2000) the addition of Al as well as of REE altered the concentrations and contents of nutrients, especially Ca and P, Fe, Mn and Si in all plant species, including *B. napus* (Table 2, Figure 1). In *B. napus* the increased nutrient concentrations most probably derive from a passive enrichment of elements relative to the decreased biomass production. In contrast, *P. sativum* showed a higher shoot biomass when supplied with Al and REE, irrespective of the element doses (Table 2). In *P. sativum* the presence of both Al and REE led to higher concentrations of P, Fe and Mn. It is reasonable that the improved growth of *P. sativum* is related to improved acquisition of elements from the growth substrate coupled with efficient detoxification mechanisms in this species (Giannakoula et al., 2008). Physiological studies could demonstrate that both Al and REE shift plant metabolism towards the production and release of carboxylates (Kataoka et al., 2002; Kochian et al., 2015) which supports element acquisition and transport from roots to the shoots.

Surprisingly, when treated with Al and REE at similar doses, shoot Al concentrations were two orders of magnitude higher than REE with a molar REE/Al ratio ranging between 2–5 µmol $mmol^{-1}$. This then indicates a higher root shoot translocation of Al compared to REE (Liu et al., 2021) and/or a more strongly pronounced sorption of REE to the peat material (Jones, 1998; Martell et al., 2004). It has to be noticed that the application of low doses of Al significantly increased the concentrations and net shoot uptake of REE, particularly LREE, in all plant species with only limited changes in shoot Al. Conversely, the addition of REE significantly enhanced shoot absorption of both Al and REE, respectively. There was no significant difference in the molar REE/Al ratios between the plant species and the treatment with both Al and REE increased the REE/Al ratios, irrespective of the treatment with either Al or REE. In the single-dose experiment, Al and REE were not supplied with the watering solutions (and vice versa). Thus, the presence of Al influenced REE accumulation more strongly than that of Al, which might suggest that the availability of REE was controlled by diffusion, rather than uptake mechanisms. Furthermore, the presence of Al might have enhanced the diffusion, uptake and translocation through changes in apoplastic pH and complexation with carboxylates (Kochian et al., 2015; Muhammad et al., 2019).

In contrast, when Al and REE were given together with the treatment solution, their uptake should be more strongly controlled by uptake than by solubility. Here, the Al concentrations remained unchanged compared to the single-dose treatment, irrespective of the concentration level and the plant species. In *L. albus*, the treatment with Al+REE did not affect REE concentrations and net shoot uptake. However, the treatment with Al+REE strongly decreased REE concentrations in *B. napus* and in *Z. mays* (Table 3, Figure 2). Thus, a reduced uptake and/or translocation may result from element exclusion through extracellular complexation (Kochian et al., 2015; Mleczek et al., 2018). Rare earth element transport in plants is mediated by Ca, K, and Na channels (Han et al., 2005). The production of carboxylates in the presence of Al, complexation of REE that form more stable complexes with carboxylates than Al (Jones, 1998) in concert with a reduction of Ca-influx (Bojórquez-Quintal et al., 2017) seem to decrease REE accumulation more strongly than that of Al which is transported by aquaporins (Wang et al., 2017). In this light, the results obtained for *Z. mays* are especially interesting because in this monocot, the application of Al and REE decreased shoot contents of REE and Ca. Dicots

generally require higher Ca concentrations than monocots (Loneragan, 1968) because of a lower concentration of cell wall pectate (White and Broadley, 2003) and their uptake systems are likely more efficient and less interfered by competing ions such as REE compared to monocots. Concomitantly in *Z. mays* the addition of Al and REE substantially increased shoot Si concentrations and contents (Table 2, Figure 1) which could be an effect of an upregulation of Si transporters in concert with enhanced Si acquisition from the growth substrate. In fact, the peat material contained only very low concentrations of Si in potentially plant available element fractions (Table 1). Therefore, the plants treated with Al and REE accessed this element pool more efficiently compared to the reference plants that grew in absence of the elements. It is reasonable that enhanced silicification might have contributed to element tolerance in *Z. mays* through co-precipitation internally or element exclusion at the root–solution interface (Barcelo et al., 1993; Wang et al., 2004; Brackhage et al., 2013). The processes involved remain a field for future studies; however, if proven, this would have major implications for our general understanding of element relationships in the soil–plant system where plants are typically exposed to Al, REE and Si in the soil solution.

The influence of Si treatment on shoot biomass and elemental composition

Similar to the single dose experiment, the application of TE (Al, Cd, and REE, without Si) did not affect the growth of *L. albus*, irrespective of the concentrations given with the treatment solution. In *Z. mays,* only at high element doses shoot development significantly declined when Cd was present in addition to Al and REE (Table 4), highlighting the high element tolerance of these species described previously (Tolrà et al., 2009). The application of Al and REE together with Cd increased the concentrations of all elements in the shoots of *L. albus* and *Z. mays* (except Al in *Z. mays*). *Zea mays* accumulated substantially higher amounts of Cd than *L. albus* and displayed similar Cd concentrations in shoots as *B. napus,* which has been described as an accumulator of Cd (Chen et al., 2018). However, in *B. napus* and *C. sativus*, the presence of low concentrations of Al, Cd and REE decreased shoot growth (Table 4), most probably as an effect of multi-element intoxication (2007) and detrimental effects on nutrient homeostasis (Page et al., 2006). In fact, in both species, the reduction of shoot growth was accompanied by altered shoot nutrient concentrations (Table 4), most likely caused by nutrient imbalances in concert with the altered shoot and root growth in the presence of the toxic elements (Kubier et al., 2019; Haider et al., 2021). The higher Ca, P, Mn and Fe concentrations in *L. albus* may indicate enhanced element acquisition through the production and release of carboxylates (Lambers et al., 2015; Lambers, 2022). Efficient nutrition allows plants to build tolerance against toxicity which may assist plants in avoiding the absorption and uptake of toxic elements such as Al, Cd and Mn, thus alleviating their impact (Sarwar et al., 2010).

The addition of Si to the plants significantly increased the Si concentrations and contents in the Si-accumulators *C. sativus* and *Z. mays* (Table 4, Figure 3). In these species, Si accumulation increased with increasing TE concentrations added (Table 4). Surprisingly, a similar effect was observed in the non-Si-accumulators *L. albus* and *B. napus* that responded with increased Si concentrations when they were exposed to high TE. However, in these species the Si contents (Figure 3) remained unchanged relative to the reference plants when TE were supplied at low doses. Possibly, in *Z. mays* and *C. sativus*, the presence of toxic elements led to an upregulation Si transport systems (Marschner et al., 1990; Liang et al., 2005; Mitani et al., 2011), while in *B. napus* and *L. albus* the increased Si concentrations are most likely through enhanced passive accumulation (Liang et al., 2005).

It should be noticed that in all plant species tested, the enhanced Si accumulation was disproportionally higher than changes of biomass, indicating enhanced Si transport to the shoots in the presence of the potentially toxic elements (Table 4, Figure 3). The enhanced Si accumulation did not relieve element stress in terms of measurable changes of biomass. Only *C. sativus* showed a slightly increased biomass in the presence of Si, compared to the TE treatment without Si, but this effect was not statistically significant at $\alpha = 5\%$. Nevertheless, the Si added substantially altered the shoot elemental composition regarding the essential and non-essential elements and this effect depended on the plant species and the concentration of toxic elements in the watering solution (Table 4, 5, Figure 3, 4). More specifically, in the non-Si-accumulating *L. albus*, concentrations and contents of Cd and REE as well as of the nutrients P, Fe and Mn were unaffected by Si. In contrast, addition of Si significantly reduced shoot concentrations of Cd and REE in *B. napus*. Da Cunha and colleagues (2008) discovered that adding Si to a Cd- and Zn-contaminated soil significantly reduced metal stress and increased biomass, which was also confirmed in our study (Da Cunha et al., 2008). In the stressed plants, shoot biomass was significantly lower in the presence of low TE doses (Table 4). Similarly, in the Si-accumulators *C. sativus* and *Z. mays*, respectively, the addition of Si decreased Cd and REE concentrations when TE was supplied at low levels (Table 4). In these species, Ca concentrations and trace nutrient concentrations remained largely unchanged, suggesting that the Si given to the plants altered the uptake and/or translocation of the toxic elements. Indeed, Farshidi (2012) and Pontigo et al. (2015) demonstrated that Si accumulators, as well as the non-Si-accumulator *B. napus,* can accumulate Si in the roots where it may form barriers and/or influence element speciation, which impacts the movement of cations during radial transport and root–shoot translocation (Barcelo et al., 1993; Liu et al., 2019). However, when TE concentrations were

high, the Si present did not influence element absorption in *B. napus* (Table 5). Possibly, the high amounts of elements exceeded the absorption/retention capacity of the barrier formed. We emphasize that the presence of Si strongly decreased Ca concentrations and increased Fe in *B. napus*, which could be an effect of biosilicification of the roots (Fleck et al., 2015), reduced Ca availability in the presence of the Si fertilizer used (Dishon et al., 2011; Bosnic et al., 2019) in concert with increased Fe uptake and translocation (Gonzalo et al., 2013; Pavlovic et al., 2013; Stevic et al., 2016; Bityutskii et al., 2018).

Unlike the non-Si-accumulators, *L. albus* and *B. napus*, the addition of Si substantially increased the concentrations of Cd and REE in the Si accumulators *Z. mays* and *C. sativus*, respectively. These species consistently showed higher shoot Fe and Mn concentrations in the presence of Si. Possibly, the Si enhanced the acquisition and transport of the transition metals Fe and Mn and consequently also of Cd in these species (Da Cunha and do Nascimento, 2009). However, changes in REE uptake and accumulation were largely independent from Ca accumulation. Calcium uptake substantially decreased in *C. sativus* in the presence of Si, while in *Z. mays,* Ca uptake increased. Since the concentrations of REE in the growth media were orders of magnitude lower compared to Ca, the different physiological responses to changes in Si supply with regard to nutrient accumulation do not seem to strongly impact REE accumulation mediated by Ca transporters (Han et al., 2005). Instead, it seems that the presence of Si and the mechanisms involved in biosilicification enhanced REE and Cd accumulation. Possibly, the Si chemically modified the apoplast (Dragišić Maksimović et al., 2012) and increased the release of metal chelating compounds (Pavlovic et al., 2013) that increased element mobility and radial transport, and/or the Si enhanced long distance transported in the form of inorganic Si-REE/Si-Cd complexes (Liang et al., 2007) and storage in silicified structures (Kameník et al., 2013). The processes involved are still enigmatic and remain field for future studies. Nonetheless, we could demonstrate that Si can decrease shoot concentrations of potentially toxic elements Cd and REE when they are present at moderate concentrations. At high concentrations in soil solution, Si enhances the shoot accumulation of Cd and REE in Si-accumulators but does not affect the accumulation in non-Si-accumulators.

Conclusion

In this study, REE had less detrimental effects on shoot growth than Al, and *Z. mays* and *L. albus* were less affected in the presence of the potentially toxic elements than *B. napus*, *P. sativum* and *C. sativus*, highlighting the higher toxicity of Al compared to REE. We emphasize that adding Al and REE increased shoot growth and improved plant nutrition of *P. sativum*, a plant species that typically lacks the ability to respond to a shortening of nutrients by increased carboxylate release. It is reasonable that the beneficial effects of Al and REE derived from a metabolic shift in *Pisum sativum* towards enhanced carboxylate production, enabling the species to cover the nutrient demands and detoxify the elements in the root zone at the same time. We could also demonstrate that *L. albus* and *Z. mays* tolerate high concentrations of potentially toxic elements (Al, REE, Cd). These plant species are promising candidates for the remediation and cultivation of acidic and polluted soils with highly mineralized soil solution. In contrast, *B. napus*, *P. sativum*, and *C. sativum* responded with declining shoot growth. The addition of Si to the plants did not restore shoot growth and consequently did not relieve plant stress. However, the addition of Si decreased shoot concentrations of the potential toxic elements in all plants species when the plants were exposed to low concentrations (10 µmol L^{-1}) in the soil solution, irrespective of their belonging to the functional group of Si-accumulators (*Z. mays*, *C. sativus*) or non-Si-accumulators (*B. napus*, *L. albus*). Moreover, when the element concentration in the growth media was high, the addition of Si strongly enhanced shoot Cd and REE concentrations and accumulation without detrimental effects on shoot biomass. Indeed, in Si treated plants of *C. sativus* and *Z. mays*, the amounts of Cd and REE accumulated were comparable to *B. napus*, which is considered a hyperaccumulator for Cd. The mechanism remains unclear. Nevertheless, we demonstrated that the role of Si in the soil–plant transfer of elements is not solely restricted to Si-accumulators. The use of Si-fertilizers could be a promising approach in phytoremediation and phytomining application enabling plant colonialization of acidic, highly Cd- and REE-polluted environments and/or fostering phytoextraction efficiency. The synergistic effects of Al and REE on (shoot) element accumulation found in this study might explain the coincidence of the REE/Al hyperaccumulation reported in the literature (Pletnev and Zernov, 2002; Fehlauer et al., 2022; Liu et al., 2022). Future studies are needed that integrate root/shoot relationships and chemical changes in the rhizosphere to elucidate the underlying processes.

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Disclosure statement

The authors report there are no competing interests to declare.

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Table 1 Total concentrations of Ca, P, Fe, Mn, Si, Al, Cd and REE (LREE: La, Nd, Ce, and HREE: Gd and Er) in the peat semi-hydroponics substrate, n=5, the numerical values represent the mean ± sd. F1: mobile or exchangeable fraction F2: acid-soluble fraction F3: fraction in oxidizable matter F4: amorphous oxide F5: crystalline oxide.

	F1	F2	F3	F4	F5
	$\mu g\ g^{-1}$				
Ca	3728 ± 192	1169 ± 223	153 ± 10:4	7.37 ± 0.62	7.00 ± 0.89
P	86 ± 33	27 ± 2.7	63 ± 7.0	8.9 ± 2.8	1.0 ± 0.5
Mn	8.4 ± 0.9	5.8 ± 0.63	2.3 ± 0.3	0.32 ± 0.04	0.11 ± 0.01
Fe	1.1 ± 0.1	1.6 ± 0.4	62 ± 14	24 ± 5.1	10 ± 4
Si	12 ± 0.7	5.2 ± 0.2	15 ± 3	3.1 ± 0.9	2.1 ± 0.2
Al	1.2 ± 0.4	3.8 ± 1.3	38 ± 6	7.5 ± 1.7	1.9 ± 0.4
Cd	0.03 ± 0.01	0.031 ± 0.002	0.011 ± 0.001	0.009 ± 0.001	0.009 ±0.003
LREE	0.036 ± 0.003	0.016 ± 0.002	0.02 ± 0.02	0.028 ± 0.004	0.022 ± 0.004
HREE	0.0013 ± 0.0004	0.002 ± 0.001	0.004 ± 0.001	0.0025 ± 0.0001	0.008 ± 0.001

Table 2 Concentrations of silicon (Si), calcium (Ca), phosphorus (P), iron (Fe), and manganese (Mn) from the dry biomass of *Brassica napus*, *Lupinus albus*, *Pisum sativum* and *Zea mays* treated with REE, Al, and Al+REE at treatment concentrations of 10 µmol L^{-1} or 100 µmol L^{-1}. The numerical data represents mean ± sd (with the following replicates: Reference: n = 5; AL: n = 3; REE & Al+REE: n = 4). Letters indicate significant differences between each treatment with the reference plants within a species ($p < 0.05$).

Species	Treatment	Biomass (g)	Si (mg g^{-1})	Ca (mg g^{-1})	P (mg g^{-1})	Fe (µg g^{-1})	Mn (µg g^{-1})
B. napus	Reference	3.9 ± 1.0a	0.8± 0.1b	25 ± 8b	4 ± 1b	109 ± 7ab	69 ± 45
	REE(10)	4.5 ± 0.7a	0.6± 0.1	33 ± 3a	6.4 ± 0.6a	133 ± 36	52 ± 10
	Al(10)	2.2 ± 1.1b	1.5± 0.1a	39 ± 11a	5.7 ± 0.7ab	141± 10a	45 ± 8
	Al+REE(10)	1.4 ± 0.4b	0.8± 0.1	35 ± 3.5a	7.5 ± 1.5a	148 ± 34a	65 ± 12
	REE(100)	2.5 ± 0.6a	0.6± 0.1	40 ± 3a	6.0 ± 0.4a	94 ± 19	59 ± 5
	Al(100)	2.4 ± 0.6ab	0.8 ± 0.1	44 ± 3a	6.8 ± 0.3a	96 ± 33b	53 ± 16
	Al+REE(100)	1.5 ± 0.6b	1.4 ± 0.3a	35 ± 1a	7.0 ± 1.1a	130 ± 27ab	73 ± 22
L. albus	Reference	3.0 ± 0.5	0.9 ± 0.1b	5.5 ± 1.3b	3.1 ± 0.4b	53 ± 4.2b	54 ± 18b
	REE(10)	2.2 ± 0.6	0.6 ± 0.1b	12 ± 1.0a	5.4 ± 0.3a	116 ± 7.2a	114 ± 36a
	Al(10)	2.7 ± 0.4	1.4 ± 0.2a	13 ± 0.3a	5.3 ± 0.4a	153 ± 38a	116 ± 36a
	Al+REE(10)	2.7 ± 0.5	0.8 ± 0.1b	14 ± 1.9a	7 ± 1a	146 ± 26a	149 ± 36a
	REE(100)	2.8 ± 0.4	1.0 ± 0.42b	13 ± 0.3a	5.0 ± 0.6a	112 ± 24a	129 ± 20a
	Al(100)	3.0 ± 0.2	0.8 ± 0.7b	13 ± 2.2a	5.3 ± 0.6a	84 ± 7.6	83 ± 5b
	Al+REE(100)	2.5 ± 0.8	1.4 ± 0.3a	12.0 ± 0.2a	5.0 ± 0.4a	114 ± 75a	104 ± 35a
P.sativum	Reference	1.3 ± 0.6b	1.0 ± 0.1a	22 ± 2a	4.2 ± 0.7b	60 ± 14c	26 ± 7b
	REE(10)	2.0 ± 0.8a	0.5 ± 0.2b	19 ± 3ab	6.0 ± 0.3a	257 ± 150a	56 ± 10a
	Al(10)	3.0 ± 0.4a	1.4 ± 0.4	17 ± 2b	4.8 ± 0.1b	84 ± 5.9 b	38 ± 2.9a
	REE(100)	2.4 ± 0.4a	1.1 ± 0.02b	18 ± 0.4b	6 ± 1a	202 ± 162a	51 ± 8.6a
	Al(100)	2.3 ± 0.3a	0.9 ± 0.1	20 ± 1a	6.3 ± 0.3a	99 ± 7.6 b	46 ± 6.5a
Z. mays	Reference	6.7 ± 1.6	0.6 ± 0.1c	10 ± 1a	3.3 ± 0.5b	70 ± 9b	56 ± 15b
	REE(10)	4.5 ± 0.9	1.3 ± 0.3b	8.9 ± 1.8ab	6.2 ± 1.4a	86 ± 7.2	95 ± 26aA
	Al(10)	5.8 ± 1.3	2.3 ± 0.8a	7.3 ± 0.4b	4.6 ± 0.6a	109 ± 18a	64± 2
	Al+REE(10)	6.3 ± 1.3	1.6 ± 0.2b	8.2 ± 1.6ab	6.0 ± 0.4aA	104 ± 32a	82 ± 17a
	REE(100)	6.8 ± 0.9	2.6 ± 0.6a	7.1 ± 1.8b	4.1 ± 0.2	102 ± 46a	62 ± 10b
	Al(100)	5.8 ± 2.1	1.4 ± 0.2b	7.8 ± 1.6b	5.2 ± 0.3a	123 ± 61a	65 ± 24
	Al+REE(100)	5.1 ± 0.4	1.4 ± 0.28b	7.3 ± 1.0b	5.1 ± 0.3a	89 ± 33	73 ± 3.6ab

Table 3 Concentrations of aluminium (Al), light and heavy rare earth elements (LREE and HREE) from the dry biomass of *Brassica napus*, *Lupinus albus*, *Pisum sativum* and *Zea mays* treated with REE, Al, and Al+REE at treatment concentrations of 10 µmol L^{-1} or 100 µmol L^{-1}.The numerical data represents mean ± sd (with the following replicates: Reference: n = 5; Al: n = 3; REE & Al+REE: n = 4). Small letters indicate statistically significant differences between each treatment with the reference plants within a species ($p < 0.05$).

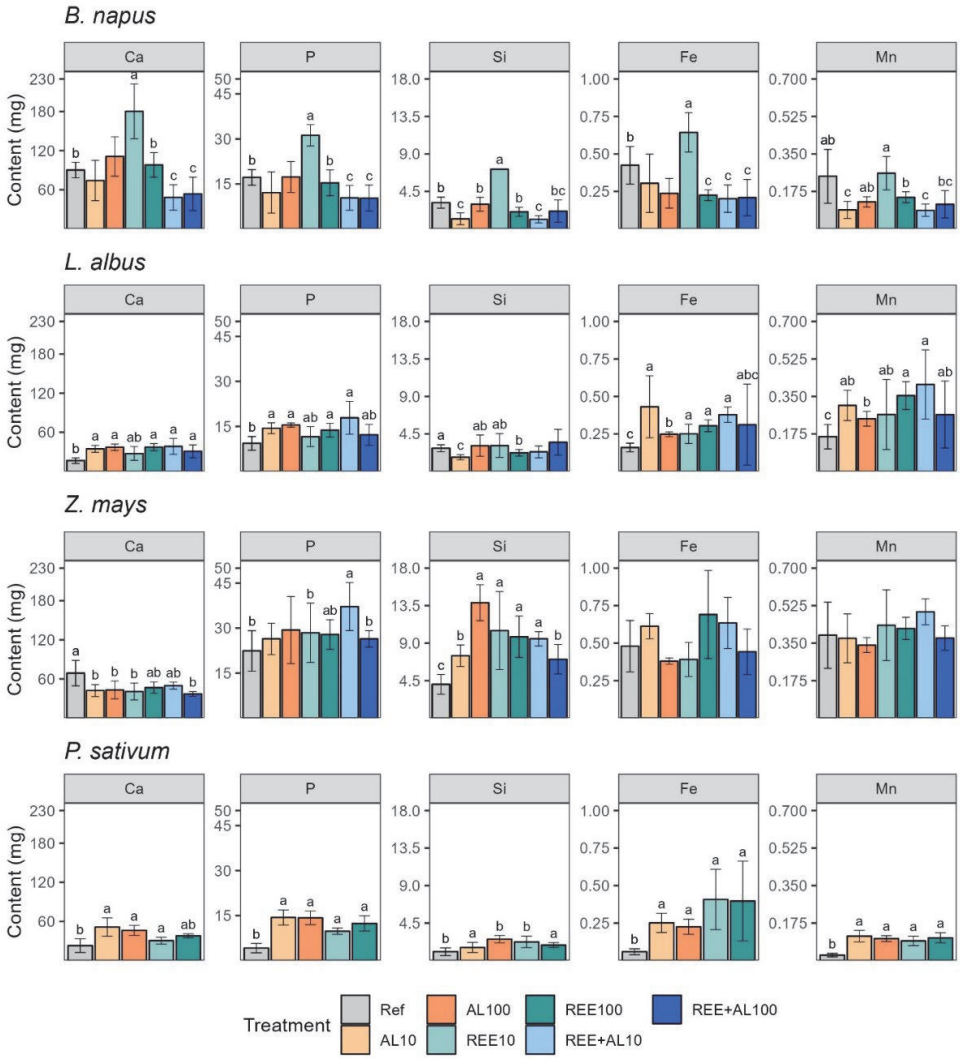
Species	Treatment	Al (µg g^{-1})	LREE (µg g^{-1})	HREE (µg g^{-1})
B. napus	Reference	53 ± 18b	0.18 ± 0.09b	0.04 ± 0.02c
	Al(10)	61 ± 6.7b	0.48 ± 0.17	0.05 ± 0.01
	REE(10)	189 ± 99a	2.9 ± 1.7a	2.1 ± 1.3aB
	Al+REE(10)	80 ± 25	1.2 ± 0.7a	0.49 ± 0.24bB
	Al(100)	90 ± 22ab	0.18 ± 0.03	0.06 ± 0.02
	REE(100)	109 ± 22a	7.7 ± 5.5aA	5.7 ± 3.8aA
	Al+REE(100)	87 ± 12	8.3 ± 2.7aA	5.7 ± 1.9aA
L. albus	Reference	27 ± 13b	0.05 ± 0.01c	0.01 ± 0.004c
	Al(10)	61 ± 12a	1.89 ± 0.62a	0.15 ± 0.08b
	REE(10)	82 ± 8.3a	0.81 ± 0.37b	0.49 ± 0.26bB
	Al+REE(10)	92 ± 49a	0.38 ± 0.05b	0.19 ± 0.06bB
	Al(100)	72 ± 5.5a	0.14 ± 0.05b	0.03 ± 0.01c
	REE(100)	71 ± 35a	4.0 ± 2.3a	2.2 ± 1.6aA
	Al+REE(100)	52 ± 24a	1.8 ± 1.4a	1.3 ± 1.0aA
P. sativum	Reference	41± 26b	0.08 ± 0.02c	0.02 ± 0.002b
	Al(10)	64 ± 12b	0.43 ± 0.17b	0.22 ± 0.12a
	REE(10)	122 ± 69a	0.51 ± 0.04b	0.27 ± 0.03aB
	Al(100)	98 ± 5.9a	0.84 ± 0.07a	0.33 ± 0.15a
	REE(100)	65 ± 13b	2.1 ± 1.3a	1.17 ± 0.60aA
Z. mays	Reference	42 ± 13b	0.08 ± 0.04c	0.02 ± 0.004c
	Al(10)	40 ± 12	0.29 ± 0.02b	0.05 ± 0.02
	REE(10)	62 ± 13a	0.73 ± 0.28b	0.37± 0.10aB
	Al+REE(10)	27 ± 3.0b	0.14 ± 0.03c	0.08 ± 0.02cB
	Al(100)	67 ± 16	0.10 ± 0.03c	0.02 ± 0.01
	REE(100)	29 ± 6b	2.1 ± 1.3a	1.7 ± 1.2aA
	Al+REE(100)	49 ± 11b	0.45 ± 0.17b	0.30 ± 0.13bB

Table 4 Concentration of Si, Ca, P, Fe and Mn in drymass of *Brassica napus, Lupinus albus, Cucumis sativus, Zea mays* for treatments TE (Trace elements: Al, Cd, REE) and TE+Si (TE + Si) at 10 µmol L^{-1} or 100 µmol L^{-1}.The numerical data represents mean ± sd for 5 replicates. Small letters indicate statistically significant differences between each treatment with the reference plants within the same species. Capital letters show differences between shoot concentrations within the same species, differentiating between treatment concentrations at α = 5%.

Species	Treatment	Biomass(g)	Si (mg g^{-1})	Ca (mg g^{-1})	P (mg g^{-1})	Fe (µg g^{-1})	Mn (µg g^{-1})
B. napus	Reference	3.9 ± 0.7a	0.8 ± 0.1b	25 ± 8b	4.6 ± 1.1b	109 ± 7a	69 ± 45
	TE(10)	2.8 ± 0.3b	0.9 ± 0.1	117 ± 19a	6.9 ± 0.2a	150 ± 55a	63 ± 1.3
	TE+Si(10)	2.2 ± 0.8b	0.6 ± 0.1cB	111 ± 32a	5.1 ± 0.6b	86 ± 9b	53 ± 16
	TE(100)	2.7 ± 0.9	0.7 ± 0.1	124 ± 21a	4.9 ± 0.7b	88 ± 18b	80 ± 29
	TE+Si(100)	2.3 ± 0.7b	1.4 ± 0.2aA	40 ± 9.6b	5.2 ± 0.6b	185 ± 77a	91 ± 18
L. albus	Reference	3.0 ± 0.4	0.9 ± 0.1a	5.5 ± 1.3b	3.1 ± 0.42b	53 ± 4.2b	54 ± 18b
	TE(10)	2.9 ± 0.7	0.8 ± 0.1	45 ± 2a	4.4 ± 0.4aB	95 ± 17a	117 ± 22a
	TE+Si(10)	2.5 ± 0.8	0.7 ± 0.1B	37 ± 3a	3.9 ± 0.24a	72 ± 10a	107 ± 40a
	TE(100)	3.1 ± 0.9	0.8 ± 0.1b	12 ± 1a	4.0 ± 0.27a	85 ± 15aB	113 ± 42a
	TE+Si(100)	3.0 ± 0.6	1.9 ± 0.7aA	16 ± 6a	5.1 ± 0.7aA	121 ± 24aA	115 ± 52a
C. sativus	Reference	7.9 ± 1.1a	0.6 ± 0.4b	18 ± 2.2c	5.2 ± 6.4	120 ± 23a	54 ± 18b
	TE(10)	4.1 ± 0.7b	0.9 ± 0.3b	119 ± 15a	4.5 ± 1.0	68 ± 9c	37 ± 13bc
	TE+Si(10)	5.5 ± 0.6b	2.5 ± 1.0aB	110± 18aA	4.4 ± 0.8	65 ± 5cB	27 ± 10cB
	TE(100)	4.0 ± 1.5b	1.2 ± 0.1b	153 ± 12a	4.7 ± 0.7	83 ± 12b	53 ± 21b
	TE+Si(100)	4.8 ± 0.7b	3.8 ± 0.5aA	30 ± 2.2bB	4.7 ± 0.6	104 ± 17aA	70 ± 15aA
Z. mays	Reference	6.7 ± 0.5a	0.6 ± 0.1b	10 ± 1bc	3.3 ± 0.4c	70 ± 10b	56 ± 15b
	TE(10)	5.1 ± 0.7b	0.9 ± 0.2b	30 ± 5aA	4.3 ± 0.6b	72 ± 13b	73 ± 10b
	TE+Si(10)	4.5 ± 1.1b	3.1 ± 1.3aA	27 ± 4aA	4.0 ± 0.4bA	70 ± 20b	81 ± 11aB
	TE(100)	4.0 ± 1.2b	1.7 ± 0.8b	9.1 ± 0.2cB	4.4 ± 0.9ab	85 ± 7b	73 ± 17b
	TE+Si(100)	4.8 ± 0.9b	4.5 ± 1.2aA	11 ± 1bB	5.3 ± 0.7aA	128 ± 76a	119 ± 26aA

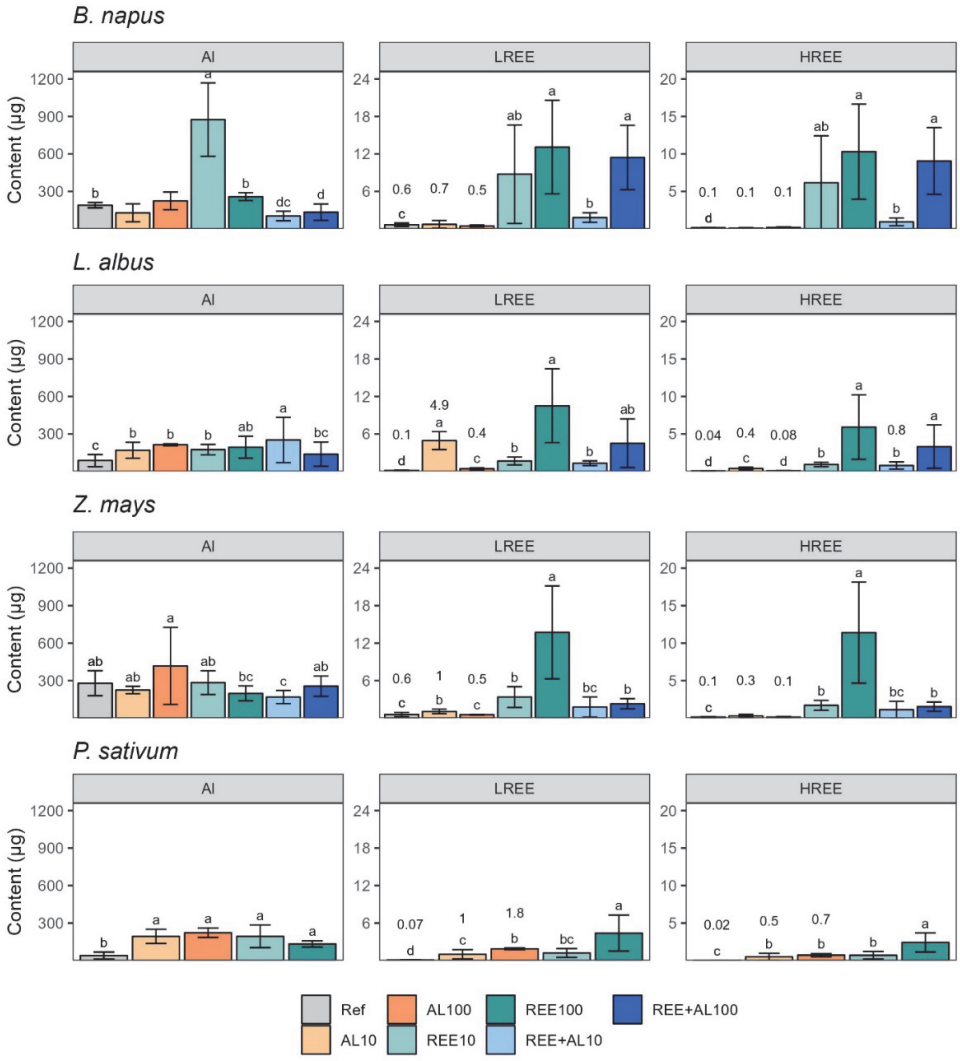
Table 5 Concentration of Al, Cd, LREE and HREE in drymass of *Brassica napus, Lupinus albus, Cucumis sativus, Zea mays* for treatmens TE (Trace elements: Al, Cd, REE) and TE+Si (TE + Si) at 10 µmol L^{-1} or 100 µmol L^{-1}. The numerical data shows mean ± sd for five replicates. Small letters indicate statistically significant differences between each treatment with the reference plants within the same species. Capital letters show differences between shoot concentrations within the same species at α = 5%.

Species	Treatment	Al (µg g^{-1})	Cd (µg g^{-1})	LREE (µg g^{-1})	HREE (µg g^{-1})
B. napus	Reference	53 ± 18b	0.3 ± 0.1c	0.18 ± 0.09c	0.04 ± 0.02b
	TE(10)	75 ± 9.3	7.9 ± 4.6bB	2.1 ± 1.3bB	1.4 ± 0.9bB
	TE+Si(10)	53 ± 12B	4.3 ± 0.5bB	0.89 ± 0.41cB	0.6 ± 0.3bB
	TE(100)	70 ± 11ab	24 ± 7aA	17 ± 13aA	12 ± 8aA
	TE+Si(100)	127 ± 34aA	38 ± 12aA	12 ± 4.8aA	10 ± 4aA
L. albus	Reference	27 ± 13c	0.11 ± 0.04b	0.05 ± 0.01c	0.01 ± 0.004c
	TE(10)	53 ± 19b	0.17 ± 0.12B	0.6 ± 0.2bB	0.4 ± 0.1bB
	TE+Si(10)	56 ± 27b	0.06 ± 0.04B	0.5 ± 0.3bB	0.3 ± 0.2bB
	TE(100)	49 ± 27b	2.3 ± 0.82bA	8 ± 3aA	6.8 ± 2.5aA
	TE+Si(100)	74 ± 32b	4.3 ± 3.0aA	8 ± 2aA	5.4 ± 2.7aA
C. sativus	Reference	48 ± 17c	0.07 ± 0.01c	0.10 ± 0.02d	0.03 ± 0.01d
	TE(10)	47 ± 5c	1.0 ± 0.24aB	1.8 ± 1.1cB	0.8 ± 0.3cB
	TE+Si(10)	74 ± 6b	0.5 ± 0.1bB	0.7 ± 0.3dB	0.5 ± 0.3cB
	TE(100)	83 ± 21b	5.8 ± 1.3bA	6.3 ± 3.9bA	5 ± 3bA
	TE+Si(100)	114 ± 36a	9.5 ± 1.1aA	22 ± 5aA	18 ± 5aA
Z. mays	Reference	42 ± 13	0.02 ± 0.01e	0.08 ± 0.04d	0.02 ± 0.004c
	TE(10)	34 ± 9	6.0 ± 1.8cB	1.2 ± 0.4aB	0.8 ± 0.2bB
	TE+Si(10)	32 ± 5	3.8 ± 1.5dB	0.4 ± 0.1bB	0.2 ± 0.1bB
	TE(100)	46 ± 18	13 ± 3.1bA	2.9 ± 1.6bA	2.2 ± 1.3bA
	TE+Si(100)	50 ± 15	24 ± 5.6aA	6.9 ± 1.3aA	5.6 ± 1.4aA



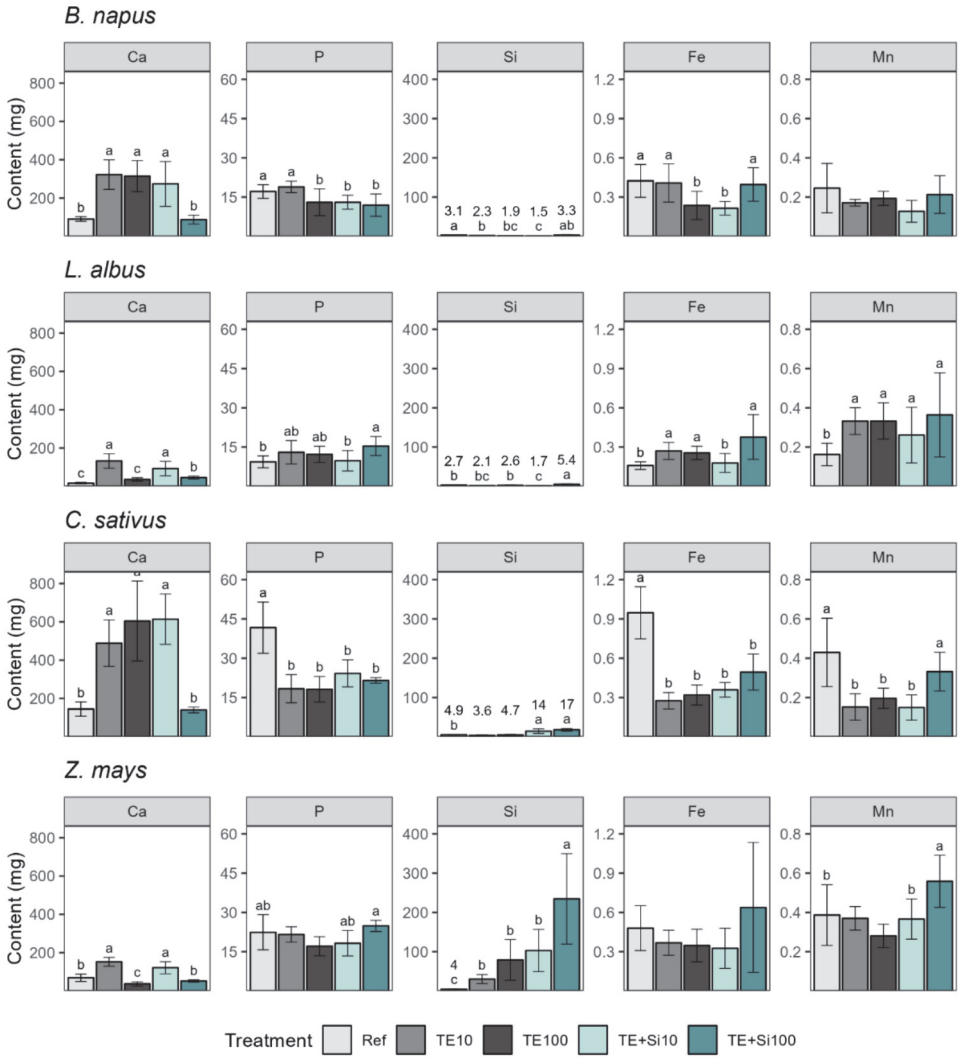
12

13 Figure 1



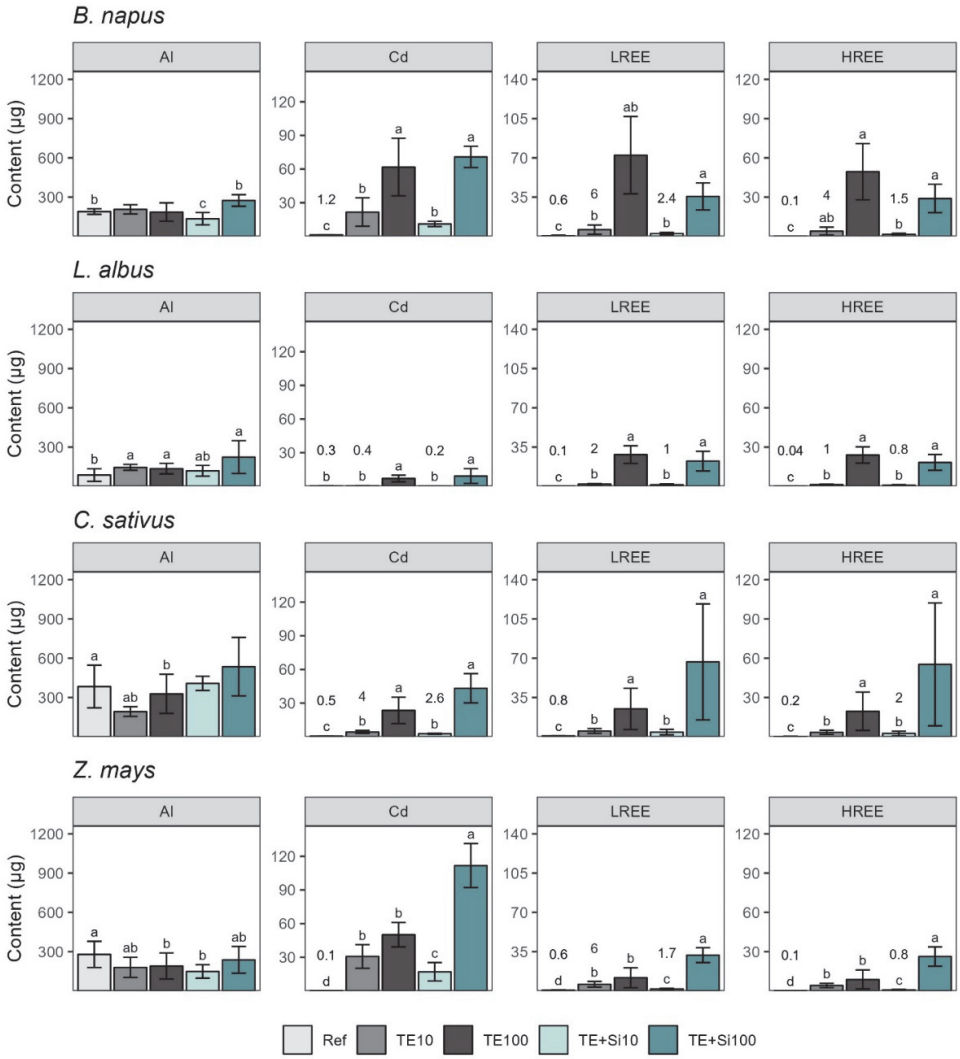
14

15 Figure 2



16

17 Figure 3



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19 Figure 4

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Figure Captions

Figure 1 Total uptake of Si, Ca, P, Fe and Mn in Brassica napus, Lupinus albus, Pisum sativum, and Zea mays, treated with AL, REE and Al+REE with the variation between treatment concentration of 10 and 100 µmol L^{-1}. The values show means ± sd of the following replicates: Reference: n = 5; Al: n = 3; REE & Al+REE: n = 4). Small letters indicate statistically significant differences between each treatment within the same species α = 5%.

Figure 2 Total uptake of Al, LREE and HREE in Brassica napus, Lupinus albus, Pisum sativum, and Zea mays, treated with AL, REE and Al+REE with the variation between treatment concentration of 10 and 100 µmol L^{-1}. The values show means ± sd of the following replicates: Reference: n = 5; Al: n = 3; REE & Al+REE: n = 4). Small letters indicate statistically significant differences between each treatment within the same species at α = 5%.

Figure 3 Total uptake of Si, Ca, P, Fe and Mn in Brassica napus, Lupinus albus, Cucumis sativus, and Zea mays, treated with TE (trace elements: Al, Cd, REE) and TE+Si (TE with Si) at a concentration of 10 and 100 µmol L^{-1}. The values are means of 5 replicates. Small letters indicate statistically significant differences between each treatment within the same at α = 5%.

Figure 4 Total uptake of Al, Cd, LREE and HREE in Brassica napus, Lupinus albus, Cucumis sativus, and Zea mays, treated with TE (trace elements: Al, Cd, REE) and TE+Si (TE with Si) with the variation between treatment concentration of 10 and 100 µmol L^{-1}, Ref = reference. The values are means of 5 replicates. Small letters indicate statistically significant differences between each treatment within the same species at α = 5%.

Appendix 2 (Other Publications)

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Coal slurry pipelines: A coal transportation method in Kalimantan, Indonesia

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ABTSRACT

This study identifies the potential economic advantages and environmental/social aspects of three different scenarios for coal slurry pipelines as an alternative to current truck haulage system in a mine in Indonesia. The developed financial model indicates that the trucks system still gives higher economic benefit compared to the three slurry pipeline scenarios tested. Nevertheless, the study results argue that there can generally be economic and environmental advantages when using coal slurry pipelines as an alternative transportation system in other countries. Hence, this study serves as a comparative analysis for when considering alternative coal transportation methods, taking into account diverse parameters.

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1. Introduction

In coal supply chains, location and transportation mode are significant economic factors as the total mine production cost is influenced by the hauling distance and transportation equipment [1]. The existence of several alternative modes of transportation such as trains, barges, trucks, conveyor belts and coal slurry pipelines, to name the most significant, can provide an option for the coal mining industry to choose the most economically efficient and effective mode of transportation [2,3].

Transportation of coal using railway is one of the modes that is widely used in several countries such as the United States, Australia, China and India [2–4]. Trucks are also often used, particularly in areas that have not been or cannot be reached by trains or water transportation [3–5]. On the other hand, sticks are an alternative way of transporting large amounts of coal, especially in watersheds. Furthermore, supply of coal through pipelines has been acknowledged since the 20th century, yet in the beginning only for relatively short distances. In the 1950s, pipeline hydraulic coal transportation served as a mature technology widely applied in underground coal mines of China [6]. The United States pioneered the coal-slurry pipeline technology and the first long-distance coal-slurry pipeline was constructed in Ohio in 1957 [7]. In recent years, the long-distance transport of coal by using pipelines has been further developed in developed countries [2,6].

In Indonesia, the use of barges is very common, such as those found on the islands of Borneo and Sumatra, which are used as coal transportation in river flows [3,8]. Coal from East and South Kalimantan is transported from the loading facility at river side by using barges directly to the consumers in Java Island [9]. Nevertheless, in Kalimantan, coal can be transported using trucks,

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conveyors, barges, or combinations of all of these modes, since usually a mine's location requires both inland and river transportation. Accordingly, trucking systems are widely used in most collieries and seem to be having a distinct advantage over long distance conveyors or pipelines due to flexibility and as road coal haulage does not need a complete infrastructure system set up before commencing the operation [5].

Despite these benefits, truck haulage is a high-cost transportation method, influenced by a high investment cost to purchase the equipment and by high operating expenses that comprise fuel, maintenance, labour and parts replacement costs [6]. Other disadvantages of truck hauling are air emissions and the environmental impact due to transport operation [2,4].

Several studies around the world have discussed the technical and economic advantages and disadvantages of all the aforementioned coal transportation methods, comparing either the systems altogether [2–6,8] or in pairs [10] for applications in several countries around the world. Further international research has been conducted on the technical and economic feasibility of coal slurry pipeline systems solely [6,11,12]. When it comes to Indonesia, however, and whilst conveyors, trucks and railway systems have been either used or technically and economically evaluated by some coal mines in the country [2,8,9,11], the potential of slurry pipelines to transport coal has not been well explored.

Based on the aforementioned, this study investigates an issue that has not been previously touched upon; the potential of a coal slurry pipeline as an alternative to trucks, for coal transportation over a relatively short transport distance for a specific coal operation in Kalimantan. The main purpose of this study is to evaluate the technical efficiency and economic feasibility of three different scenarios of coal slurry pipeline systems to be applied on the coal mining project under evaluation and compare results with the already established transportation system of trucks. Environmental and social aspects of the potential application of coal slurry pipelines in Indonesia are also evaluated in this study.

1.1. The subject coal mine

The coal operation under investigation is an open-cut coal mine located in South Kalimantan region, Indonesia. Due to confidentiality issues, the real identity of the mining project or the owner company, as well as some details on the conceptual plan of the pipeline hydraulic design cannot be published in this study. Nevertheless, several important technical data and information are discussed in this study to support the technical and economic assessment of the coal slurry pipeline system. Most of the fixed values of parameters and assumptions made were addressed from the owner company.

Hence, the coal operation is named as the 'Subject Mine' hereinafter. Operations in the Subject project started in 2006; the mine has a footprint of 24,100 ha and has a permit to operate until 2036. Currently, the mine has capacity to produce 10 million tonnes per annum of thermal coal for both domestic and export markets. The company is planning to increase production to a maximum target of 34.6 million tonnes per annum in 2024. Accordingly, the coal transportation system will need to be re-designed and adjusted to the new production capacity conditions.

Similar to many collieries in Indonesia, the Subject operation employs direct truck haulage from mine to port stockpile and haulage via a run of mine (ROM) stockpile to port. The ROM crusher has a capacity of 700 tonnes per hour and is able to operate 6,000 hours annually. This allows 4.2 million tonnes of crushed coal production to be transported to the port. The remaining Subject's uncrushed coal production is transported by trucks and will not be analysed in this report. At the port, the coal is loaded onto barges and moved to a transshipment point.

The calorific value of the coal product is 5,300 kcal/kg in air dried basis. For marketing purposes, the maximum allowable percentage of moisture and ash content are 37% and 7%, respectively. The marketable coal particle size is set at 50 mm maximum. There is a minimum size limit of 3 mm.

1.2. Slurry pipeline systems

Compared with fluids transportation, either liquid or gas, solids transportation using pipeline is usually more challenging and more expensive. However, pipeline utilisation can be the most economic mode to transport the solid materials for many cases over both short and long distances [12].

For a number of decades in the mining industry, slurry pipeline application has been beneficial to transport coal, iron, copper, phosphate, limestone and other minerals in the form of concentrates, aggregates, or tailings.

The earlier long coal slurry pipeline is the Black Mesa pipeline in the United States, built in 1970. The pipeline was able to transport 5 million tonnes of coal per annum using a 457 mm pipeline over 439 km of distance from Arizona to Nevada [7,11,13]. The World's longest slurry pipeline started in 1979 and is at Etsi, also in the United States. It has a total length of 1,036 km and a transportation capacity of 25 million tonnes of coal slurry per annum [7]. Furthermore, at Kudremukh in India there is one of the most successful slurry pipeline systems, being 57 km long inside which iron ore slurry is being transported [7].

In 1986, Bertram and Kaszynski [14] summarised the available coal slurry pipeline systems that were aligned in comparison to the main objectives that should be achieved and the required transport distance. The summary of the study provides information about how the best pipeline alternative depends on the end use of coal product. The systems are conventional fine coal, conventional coarse coal, coal-water mixture, and stabilised flow. That list was also distinctly in line with the study later conducted by Shook & Roco [15]. Jacobs [16] reported that dilute phase, sliding bed and stabilised mixture are three main alternatives of slurry system that are suitable to transport coarse particles in horizontal flow. Moreover, a new coal hydro-transport method using heavy media is worth consideration for the transportation of export size coal from the mine. The liquid, which density is higher than that of coal, allows the transportation of lump coal without any requirement for a complex dewatering process [11]. The other benefits of this method are lower energy and water requirements.

Compared to coarse coal pipeline system, the fine coal pipeline may have the potential to outperform due to a constraint to transport coarse coal in short distance without any risk of blockage [17].

1.2.1. Technical aspects of coal slurry pipeline

Several factors should be considered in coal slurry pipeline design, such as slurry velocity, rheology, slack flow, concentration of the solid in slurry and other slurry characteristics, hydraulic grade, size distribution of the coal particles, tank level and transport vehicle properties [18,19]. These factors, in addition to pipe wall roughness, pipeline hydraulic design, and other pipeline system characteristics, particle shape, and friction coefficient, are acknowledged by Gillies [20], to have significant effect on the pressure drop and energy consumptions of pipeline systems.

The pipeline diameter influences fluid dynamics principles, operating ranges and economic justifications [21], and is determined by the slurry throughput target [22].

Run-of-mine (ROM) coal is classified as a mixed flow slurry because of its largest particle size distribution. Particles might be as large as 50 mm and the size distribution ranges from non-settling to settling behaviour in which the finer particles may modify the transport liquid properties by increasing the mixture viscosity [16]. Thus, the Subject mine's coal product size has a similar wide range that might affect the slurry properties and behaviour. In terms of the relationship between particle size and slurry properties, controlling particle size distribution is important for obtaining efficient packing density, as a maximum packing density means less void between coal particles which leads to lower water needs and higher viscosity [23].

Possession of knowledge about coal slurry rheology is important to determine coal slurry pipeline technical application such as preparation, pumping system, pipelining, and mixing system

[24]. The slurry rheology depends on solid particle shape, solid size distribution, and volumetric concentration [15]. This is also supported by other studies which concluded that in coal water mixture, rheology properties are controlled by solid volume fraction, coal physical properties, particle size distribution, type and amount of additives, addition of electrolyte, and temperature of the slurry mixture [23].

The increase in particle degradation rate will increase slurry viscosity and pressure drop; therefore, it is important to consider the effect of coal degradation during pipeline system designing process [25]. Collisions due to particle – particle and particle – boundaries interactions might occur in pipe, pumps, bends, valves, and during the loading or discharging points of the pipeline system. The rate of particle fracture might increase when the collision occurs at high relative velocities [15]. As coal is a rather brittle material, the degradation can be notably severe; however, higher magnitude of solid concentration as big as 50–60% weight might reduce the degradation rate [16].

In terms of slurry flow regime, the research by Duckworth *et al.* [17] showed that coarse coal with maximum size of 20 mm may be transported under laminar flow in a 150 mm diameter pipe. The coarse coal fraction ranged from 0 to 0.48 mm with total concentration of coal at 53–67%. The laminar flow could be reached if the fluid carrier with non-Newtonian behaviour has sufficient yield stress to support the largest particle that has to be transported.

Another aspect that needs to be considered when designing slurry pipeline system is deposition velocity. Deposition velocity is the velocity at which deposition of solid particles occurs in the flow. It is important to precisely know this velocity in order to set a minimum operating velocity of the slurry pipeline. The lowest speed of slurry velocity is usually kept at 0.5 m/s above the deposition velocity [26]. Deposition velocity magnitude is controlled by particle size, material density, inner pipe diameter and solid concentration. In 1970, Traynis [27] developed equations that are used to calculate critical velocity. This critical velocity has to be maintained to prevent the solid particles from being accumulated at the bottom part of the pipe. The critical velocity V_{cr} can be calculated using Equation 1 by Traynis [27,28] below.

$$V_{cr} = (gD)^{1/2}\left[\frac{(\rho_s - \rho_{hm})/\rho_s}{f_{DL}\ k\ C_D}\right]^{1/3} \tag{1}$$

where:

g: is the gravitational acceleration
D: is the pipe inner diameter
ρ_s: is the density of the solids
ρ_{hm}: is the density of the slurry
f_{DL}: is the Darcy friction factor
k: is the permeability co-efficient (constant for coal)
C_D: is the drag co-efficient of particles for heterogeneous slurry

To calculate the frictional gradient i_m the formula is shown in Equation 2 [27,28]:

$$i_m = i_L\left\{1 + C_v\left(\frac{\rho_s - \rho_L}{\rho_L}\right) + \left[\frac{(gD)^{1/2}}{k}\cdot\frac{C_{vc}(\rho_s - \rho_{hm})}{C_d V \rho_L}\right]\right\} \tag{2}$$

where:

i_L: is the hydraulic gradient
C_v: is the concentration of solids by volume
ρ_L: is the density of the liquid carrier
C_{vc}: is the critical concentration of solids by volume
V: is the mean flow velocity

The hydraulic gradient i_L is calculated using the Darcy-Weisbach equation as shown in Equation 3.

$$i_L = \frac{f_{DL} V^2}{2gD} \tag{3}$$

1.2.2. Cost of coal slurry pipeline

Coal slurry transportation, for instance the coal water fuel (CWF), requires additional expenditures that are generated by the needs of complex and high-energy preparation, capital expenditures, operational cost for wear material and surfactant. The excessive costs for slurry transportation can be compensated for if the transport distance is more than 500 km in comparison to rail [29]. Other studies have also compared pipeline transportation of coal to rail transportation of coal and their results showed that slurry pipeline transportation offers low cost benefit. The study that was conducted for a coal shipment distance greater than 1600 km indicated that the advantages of coal slurry transportation required lower labour force and transport cost [30].

A study by Lesmana and Hitch [11] found out that coal slurry pipeline for a short distance of 8 km had potential benefits due to mine's terrain conditions that allowed the utilisation of gravity flow. The mine's terrain conditions resulted in lower pumping and energy requirements. Transport cost via slurry pipeline depends on operational factors to reach its optimum benefit; such as compatibility of production rate and pipe diameter size. As a function of target annual throughput, pipeline diameter size will give different economical value for a particular production rate.

2. Methodology

2.1. Technical analysis

Calculations for pipeline hydraulic design acknowledged the pipeline throughput requirement based on production rate, coal particle size, coal end use, and coal transport distance of the subject mine. Due to confidentiality, not all technical details are given in this study, while some parameters, assumptions, constant values, and limitations that have been addressed from the mining company are discussed hereinafter.

Even though there is no exact limit for minimum particle size of the coal product, it is preferable that the coal particles should not be too fine to avoid excessive dewatering costs. Because this study did not cover a detailed analysis of fine particle attrition and its recovery cost, it is assumed that fine particles produced during slurry transport will not be a commercial product of the Subject mine. The moisture content of coal product is assumed to meet the maximum limit that was set for the market (TM of 35.7%). In order to achieve the total moisture limit, dewatering process was included in the overall slurry pipeline process.

The slurry pipeline is assumed to have a constant horizontal flow; thus, the losses in the pipe bends, fittings, valves, and static head were not included in the calculation even though it will be necessary to include those losses in a later more detailed design. In a horizontal flow, the main cause of pressure drop or hydraulic gradient is the friction along the pipe. Three scenarios of liquid carrier in the slurry pipeline that are analysed in this study are:

(A) Scenario 1: Water with 30% of coal fines in the total weight concentration of coal fraction in the slurry. This assumption is based on test loop conducted by Wilson *et al.* [31] which showed that 30% of coarse particle will degrade along the pipe. The fines produced during particle attrition in the pipe are considered to have an effect to the slurry viscosity.

(B) Scenario 2: Viscous liquid with physical properties of glycerine. The liquid carrier is expected to have sufficient viscosity that allows good stability of the coarse particle flow. One of the alternative methods is by using liquid that is derived from palm oil waste due to its abundance in Kalimantan. Hydrolysis of palm oil waste empty fruit bunch (EFB) using dilute sulphuric acid and phosphoric acid as catalyst was found to be able to produce

fermentable sugars [32]. For simplification, the liquid is assumed to have similar properties with glycerine. Then, the assumed density and dynamic viscosity for the liquid are 1,260 kg/m^3 and 0.9 Pa, respectively.

(C) Scenario 3: Mixture of coal water slurry (CWS) and coal sewage sludge (CSS). For Scenario 3, the liquid carrier will be a mixture of CWS and CSS with a ratio of 100:10. Based on a research conducted by Li *et al.* [33], the CWS and CSS mixtures will have apparent viscosity of 2 Pa at maximum shear rate of 180 s^{-1}. This condition could be achieved with solid total weight concentration of 60%. Thus, in Scenario 3, the density of the liquid was assumed by calculating the relative density of a mixture with 'CWS and CSS solids loading of 60% by weight' [33].

The volumetric concentration C_v was calculated using Equation 4 :

$$Cv = \frac{S_f C_w}{\left[S_S - \left(S_S - S_f\right) C_w\right]} \tag{4}$$

where:

S_f: is the liquid relative density

S_S: is the coal relative density

C_W: is the concentration of solids by weight

Then, using the calculated volumetric concentration C_v and flow rate of the solid Q_S the flow rate of the mixture Q_m was calculated in Equation 5 by:

$$Q_m = Q_s / C_v \tag{5}$$

To determine the slurry dynamic viscosity of mixtures with high solid volume concentration, otherwise the corrected dynamic viscosity of the liquid, the equation by Thomas (1965) was used [34], as shown in Equation 6.

$$\mu_r = 1 + 2.5C_v + 10.05C_v + 0.00273e^{16.6Cv} \tag{6}$$

The kinematic viscosity can then be calculated as follows in Equation 7:

$$\mu_s = \mu_r \mu_l \tag{7}$$

where:

μ_r: is the corrected dynamic viscosity of liquid (after fines)

μ_l: is the dynamic viscosity of liquid

Then, the mean flow velocity V_m of the slurry can be estimated by calculating the Reynolds Numbers with formula [34] as shown in Equation 8:

$$Re = \frac{\rho_m \, Vm \, D}{\mu_s} \tag{8}$$

where:

ρ_m: is the density of the mixture

D: is the pipe diameter

For a Reynolds number below 2,000, the flow occurs in laminar regime and the friction factor f_D is independent of the pipe roughness. Therefore, the Darcy friction factor is given as shown in Equation 9.

$$f_D = Re/64 \tag{9}$$

In fully developed turbulent flows (Re > 4,000), the pipe roughness determines the friction factor. The Swamee-Jain formula (Equation 10) is an approximation of the implicit Colebrook-White equation and is suitable to calculate the Darcy-Weisbach friction factor for the range of Reynolds numbers from 5,000–100,000,000 [34].

$$f_D = \frac{0.25}{\left\{ log_{10}\left[\frac{\varepsilon/D}{3.7} + \left(5.74/Re^{0.9}\right) \right] \right\}^2} \tag{10}$$

where:

ε: is the pipe surface's roughness height

The required power for slurry pumping is a function of density ρ, volumetric flow rate Q, friction factor f, pipe diameter D, and total pipe length L. The formulae to calculate the required pumping power W_{pump} are shown in Equations 11 and 12 [31].

$$W_{pump} = \frac{8\rho Q^3 f}{\pi^2 D^5} L \tag{11}$$

$$\eta = \frac{W_{pump}}{W_{in}} \tag{12}$$

where:

W_{in}: is the input power

η: is the pump efficiency

2.2. Financial estimation

The economics of coal slurry pipeline were analysed using cost simulation for life-of-mine (LOM) scenarios. Due to a limited access to get the real data of the mining costs, financial analysis for truck system and all pipeline system scenarios used the same cost estimation obtained from analysis by Meister [35] for Indonesia sub-bituminous coal. As the estimated cost by Meister is based on 2008 economic conditions and this report is developed by referring to 2017 conditions, thus the 9-year difference is compensated for by a multiplying factor. The multiplying factor of 1.42 is based on a 4% inflation rate assumption for 9 years [36].

2.2.1. Truck costs

Costs of truck transport only consisted of operation expenditures due to the current coal hauling scheme applied to the Subject mine. The company hires local contractors to provide coal hauling services; thus, the coal hauling cost is an all-in cost that has already covered labour, fuel, truck maintenance, and other wearable materials costs. The hauling cost is expressed in a unit of measurement that represents this cost for the transport of one tonne over one kilometre US $/tkm; therefore, cost variation due to any distance change is taken care of. The hauling road maintenance is not covered in the contract agreement, therefore, hauling road maintenance cost will be added in the calculation. The hauling cost and maintenance cost of a mine can be seen in Table 1 [36].

2.2.2. Coal slurry pipeline costs

Pipeline cost was divided into capital expenditures (CAPEX) and operating expenditures (OPEX). The estimated capital cost components consisted of expenditures for slurry preparation

Table 1. Trucks system cost.

Item	Value	Unit
Hauling cost	0.0923	US$/tkm*
Road maintenance cost	0.0153	US$/tkm

Source [36]:.

A tonne-kilometre (tkm) is a unit of measurement that represents the transport of one tonne over one kilometre.

Table 2. Pipe supply and installation indicative cost.

		Cost (US$/unit)		
Description	NPS mm unit	250	300	400
Plain steel standard wall (API 5LB ERW)	m	76.21	92.97	124.98
HDPE (Class 9) (SDR11 PN 16)	pipe m	25.15	40.39	64.78
	Pipe installation m	380.28	467.16	765.13

Table 3. Pump supply and installation indicative cost.

	Indicative cost ('000 US$/unit)		
Power (kW)	500	750	1000
Centrifugal slurry pump	77.73	108.98	114.31
Positive displacement plunger pump	713.31	1,070.73	1,427.38
Positive displacement diaphragm pump	1,362.61	2,037.05	2,724.45
Pump installation – in mine site	1x pump supply cost		

facilities, an assumed 22 km long pipeline, pumps, and mixing and dewatering facilities. An extra grinding equipment was added to Scenario 3 for CWS preparation. All CAPEX were considered and no salvage value at the end of the infrastructure or equipment life time. The depreciation and amortisation calculation were assumed to be straight line method only for mixing tank and dewatering plant, pumps, land acquisition, and the initial installation expenditures. Expenditures to purchase the pipes cannot be depreciated as the pipeline lifetime is less than a year.

The capital expenditures to build the pipeline and pump system (Tables 2 & 3 respectively) were derived from the *AusIMM Cost Estimation Handbook* [37]. Due to the costs having being calculated in Australian dollars (AUD) in this handbook, a currency convertor index was used to turn the costs into U.S. dollars (US$); one US$ was considered equal to 1.31 AUD (2017 average prices, based on data derived from bloomberg.com).

The operating costs consist of power supply, liquid carrier, and preparation cost. In Scenario 3, additional grinding cost was added to cover the cost required for fine coal preparation.

3. Results

In this section, the results of the proposed methodology are discussed, having applied constant values, limitations and assumptions made appropriate for the developed pipeline hydraulic design. Some of these values and limitations are products of confidential discussions with the company that runs the Subject mine, while others are derived from the international literature by applying certain conditions and limitations. Further key results that are illustrated on the tables discussed hereinafter have been calculated using the equations described in the previous sections.

3.1. Technical analysis result

Based on these it is assumed that fine particles cannot be sold. So that, in Scenario 1, the amount of saleable coal is only 30% of the actual production. In Scenarios 2 and 3, the saleable coal is assumed to be 95%, which means that only 5% fine particles were produced during the slurry pipeline

transportation. Furthermore, for the production of CWS – CSS mixture, Scenario 3 is assumed to require another 3% of fine coal.

The hauling target for slurry pipeline is 4.2 million tonnes per annum (tpa), based on current production or crushed coal as described in the introductory section. This target was set so that the slurry pipeline will only transport crushed coal and there will be no CAPEX required to build a preparation plant.

It is assumed that plain steel pipeline and HDPE pipeline will be used to transport the coal and the liquid carrier, respectively. The plain steel pipeline is commonly used in slurry service because of its toughness quality, weldability and relatively low cost [31]. Centrifugal pump will be used in Scenario 1 to transport the coal and in all scenarios, to transport the liquid carrier. In slurry operation with a fixed speed pump but without valve to control the flow, steadiness of laminar flow is possible only if there is a tight control of slurry consistency [31]. Therefore, laminar flow in Scenarios 2 and 3 stability has to be maintained with a stable pump. Positive displacement pumps are known to be more unconditionally stable than centrifugal pump [15,16]. Thus, Scenarios 2 and 3 will use positive displacement pumps to maintain the stability of the flow.

The results of hydraulic parameters calculations are shown in Table 4. For the hydraulic parameters of the pipeline system that are used to recirculate the liquid carrier back to the ROM stockpile, the respective results are illustrated in Table 5.

3.2. Financial analysis result

The basic cost assumptions for the financial model are illustrated in Table 6, while the following boundary conditions have also been taken into account:

- Economic parameters based on 2017 financial conditions and regulations
- Coal price assumption referred to Harga Batubara Acuan [36] that is regulated by Indonesia Ministry of Energy and Mineral Resources for coal with calorific value of 4,000 kcal/kg GAR.

Table 4. Hydraulic parameter of coal slurry pipeline.

			Slurry pipeline		
Parameter	Symbol	Unit	*Water (with 30% fines)*	*Glycerine-like liquid*	CWS – CSS
Production		tpa	4,200,000	4,200,000	4,200,000
Saleable coal		tpa	2,940,000	3,990,000	3,862,231
Distance		km	22.00	22.00	22.00
Concentration of solids by weight	Cw		0.50	0.50	0.50
Coal relative density	Ss		1.30	1.30	1.30
Liquid relative density	Sf		1.00	1.26	1.16
Concentration of solids by volume	Cv		0.43	0.49	0.47
Specific gravity of the mixture	Sm		1.13	1.28	1.23
Pipe diameter	D	m	0.25	0.40	0.40
Flow rate mixture	Q_m	m^3/s	0.24	0.21	0.22
Mean flow velocity of mixture	V_m	m/s	4.80	1.66	1.73
Dynamic viscosity of liquid	μ_l	(Pa·s)	0.0009	0.90	2.00
Corrected dynamic viscosity of liquid (after fines)	μ_r	(Pa·s)	1.5209	1.6199	1.5836
Kinematic viscosity	μ_s	(Pa·s)	0.0014	1.4579	3.1672
Reynold Number	Re		918,832.14	575.55	257.62
Friction factor	f_{DL}		0.0153	0.1112	0.2484
Hydraulic gradient	i_L	m/m	0.0717	0.0389	0.0947
Frictional gradient	i_m	m/m	0.0824	0.0398	0.1026
Deposition velocity	V_{cr}	m/s	3.30	1.09	1.27
Power required for pumps	W	kW	3,779.04	2,212.78	5,233.78
Efficiency		%	75.00	75.00	75.00
Required pump power		kW	5,038.72	2,950.37	6,978.37
Pump power		kW	750.00	1,000.00	1,000.00
Number of pumps			7	3	7

Table 5. Hydraulic parameter for liquid carrier pipeline.

			Slurry pipeline		
Parameter	Symbol	Unit	*Water (with 30% fines)*	*Glycerine-like liquid*	CWS – CSS
Liquid density	ρ	kg/m^3	1,000.00	1,260.00	1,160.71
Area	A	m^2	0.07	0.07	0.07
Volumetric flow rate of the liquid	Q	m^3/s	0.13	0.11	0.11
Mean flow velocity	V	m/s	1.88	1.49	1.62
Dynamic viscosity of the liquid	μ	(Pa·s)	0.00089	0.90	2.00
Reynold number	Re		634,843.64	627.79	282.51
Hydraulic gradient	i_L	m/m	0.0076	0.0387	0.1013
Fanning friction factor	f		0.0126	0.1019	0.2265
Pressure drop	Δp	kPa	1,632.59	10,522.95	25,384.60
Power with efficiency 75%	W_{pump}	kW	217.83	1,114.29	2,917.95
Pump efficiency	η	%	75.00	75.00	75.00
Input power	W_{in}	kW	290.43	1,485.73	3,890.60
Selected pipe		m	0.30	0.30	0.30
Selected pump		kW	500.00	750.00	1,000.00
Number of pumps			1	2	4

Table 6. Cost assumptions for financial calculations.

Cost assumptions	Value	Unit
Mining cost	13.95	US$/t
Crushing and preparation	2.56	US$/t
Grinding cost	2.00	US$/t
Port and loading	2.56	US$/t
Tax	45.00	%
Royalty	13.50	%
Coal price	40.00	US$/t
Liquid price	600.00	US$/t
Power consumption	7.00	kwh/m^3
Power consumption for coal	25.00	kwh/m^3

- Pipeline economic lifetime = 8 months.
- Expected annual coal price increase at 2% per annum.
- Interest and inflation rate at 5 and 4% respectively [38].
- Discount rate at 12% based on WACC calculation.
- All rates are flat over the life of mine.

Following the necessary calculations, the detailed comparison of financial results for each one of the three scenarios is illustrated in Table 7.

Table 7. Economic comparisons.

			Slurry pipeline		
Parameter	Unit	Trucks	Water (with 30% fines)	*Glycerine-like liquid*	CWS – CSS
Total revenue	US$	1,839,553,128	1,705,575,472	1,834,954,245	1,691,613,876
Total OPEX	US$	(1,368,599,545)	(1,276,835,680)	(2,343,155,333)	(1,350,754,549)
Total CAPEX	US$	N/A	(80,897,799)	(135,340,098)	(185,903,305)
NPV	US$	148,876,912	81,108,604	(357,685,503)	3,030,843
IRR	%	N/A	70	N/A	14

4. Discussion

From the determination of the parameters in Tables 4 & 5, the cost assumptions in Table 6, and the economic results and comparisons that are eventually presented in Table 7, some remarkable findings are brought forward. Accordingly, a detailed analysis of some of the most critical technical and economic factors is made hereinafter.

4.1. Technical analysis

4.1.1. Slurry flow regime

In Scenario 1, the occurrence of 30% fines due to particle attrition cannot cause a significant increase of the liquid viscosity. The liquid carrier is expected to cause the slurry to flow in turbulence with a Reynolds number of 918,832, which means that the liquid carrier cannot provide a stable slurry. The turbulent flow, roughness of pipe material and pipe inside diameter will have influence on the friction factor.

Laminar flow is predicted to occur in the slurry pipeline system in Scenarios 2 and 3. The usage of more viscous and high-density liquid would allow a more stable flow in the pipeline. The Reynolds numbers for Scenarios 2 and 3 are 576 and 258, respectively. This laminar flow will still occur even though there is a change of pipe diameter until 0.1 m for both scenarios.

4.1.2. Pipeline diameter

It is important to keep the mean flow velocity above the critical velocity value to reduce the risk of solid deposition or blockage in the bottom part of the pipe. Since the volumetric flow rate has to be fixed with the production target, the pipeline diameter becomes the variable that can be modified. From the simulations performed during the study, the maximum pipeline internal diameter values for Scenarios 1, 2, and 3 are 0.25, 0.4, and 0.4 m, respectively. In Figure 1, it is shown that by increasing the pipeline diameter, the mean velocity of the flow is reduced, but at the same time the deposition velocity increases.

4.1.3. Critical velocity as a function of liquid viscosity

The slurry in Scenario 1 has to operate at a velocity above 3.3 m/s; meanwhile in Scenarios 2 and 3, the slurries' deposition velocities are 1.09 m/s and 1.27 m/s, respectively. Scenario 2 has the lowest deposition velocity as it has the largest slurry density among others. This confirms the relationship between liquid carrier density and its viscosity and the magnitude of deposition velocity. Because

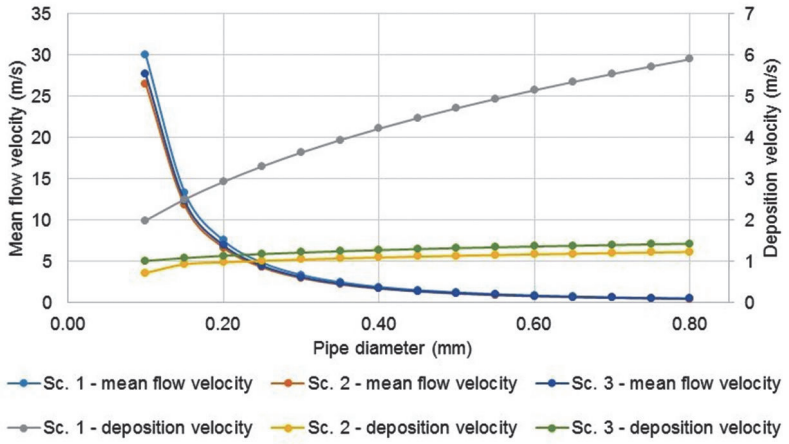

Figure 1. The effect of pipe diameter changes on mean flow velocity and deposition velocity.

the solid density for all scenarios is the same, the liquid density becomes the critical variable that makes the three slurries having different densities. Accordingly, the analysis showed that higher density and viscosity of the fluid carrier will decrease the deposition velocity.

4.1.4. Hydraulic gradient and pumping power

Scenario 3 has the highest hydraulic gradient and pumping power requirement due to the high viscosity of the liquid carrier, which leads to low Reynold numbers and affects the friction factor calculation. Because the system has high friction factor, the hydraulic and frictional gradient become high, which consequently requires a high pumping power.

In Scenario 1, the relatively high hydraulic gradient and frictional gradient values are caused by the high mean flow velocity and small size of the pipeline diameter. On the other hand, Scenario 2 has the lowest hydraulic and frictional gradient and required pumping power among all the pipeline system scenarios due to combination of low friction factor, low mean flow velocity, high diameter (higher than in Scenario 1 but equally high to the diameter in Scenario 3) and the higher liquid density. The required pumping power is influenced by the change of pipe diameter and overall pipe length as shown in Figure 2a & b.

More precisely, Figure 2a shows that the change of pipe diameter has the least effect on Scenario 1 because the high Reynolds number leads to low friction factor in this case. The change of pipe diameter significantly affected the slurry in Scenario 3, followed by Scenario 2. In Figure 2b it is shown that Scenario 3 is highly affected by the overall pipeline length, followed by Scenario 1 and Scenario 2. These results are in accordance with frictional and hydraulic gradient of each system. For pumping of the liquid carrier, Scenarios 2 and 3 which use more viscous and heavier liquid will require more pumps with higher power than Scenario 1.

4.2. Financial analysis

When it comes to the application of the developed financial model in this study, the most significant outcome has to do with the fact that the already established trucks system still gives the highest benefit for the company. This is confirmed by the highest Net Present Value (NPV) comparison in the three scenarios of slurry pipeline. Among all the slurry pipeline scenarios, only Scenarios 1 and 3 result in a positive NPV for the project, while Scenario 2 concludes to a negative NPV.

Based on all assumptions used in this study, the trucks system performs almost two times higher than Scenario 1, mainly because the trucking cost at the Subject mine is considerably low. The low trucking cost in Indonesia might be due to low labour costs in general, whereas this might be an issue in other, more developed countries such as the United States or Australia.

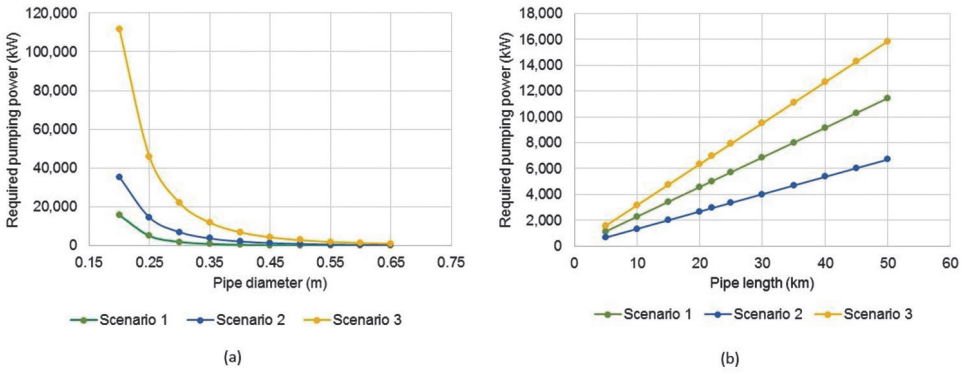

Figure 2. Relationship between (a) pipe diameter and required pumping power (b) pipe length and required pumping power.

Moreover, the cost of a new slurry pipeline system will only be competitive where there is a need for new construction. Otherwise, if the required infrastructure and equipment are already available, the cost associated with a new slurry pipeline construction might cause the pipeline system to be unfeasible [39]. This reason seems to be the fundamental factor that has caused the pipeline scenarios not to be as economic as the truck transportation system in this study.

Scenario 2 gives the lowest NPV value and the highest operational expense due to high cost of the liquid. However, this high operating cost might be overestimated because originally, the idea is to use liquid from palm oil waste EFB. The actual cost of the liquid might be lower than the assumption used in this study.

Since this research was conducted as a preliminary study, most of economic variables are based on assumptions. The cost assumptions usually possess some error which can occur as over-estimation or under-estimation. Therefore, a sensitivity analysis is important to determine how values variance of variables will affect the result of economic calculation. The sensitivity analysis applied in a number of important parameters provides general information of which the variable is the most sensitive factor such that its change might cause significant impact on the economic value of the project.

4.2.1. Coal price

Due to the coal price being rather volatile, a sensitivity analysis on the coal price proved to be necessary as shown in Figure 3a. Even though the coal price variation does not affect the order of profitability of the transportation systems, it seems to have a notable impact on the calculated NPVs. Given a 10% in coal price, the NPV of Scenario 3 will be positive, whereas the value in Scenario 2 will still not be able to be positive even when there is a 25% coal price increase.

4.2.2. CAPEX and OPEX

In Figure 3b it is illustrated that capital expenditures variations significantly affect the NPV of Scenarios 1 and 3. If there is a CAPEX decrease as this was assumed in the financial model, the NPVs of the pipeline systems tend to increase at a bigger rate compared to the respective NPV of the trucks system. The value in Scenario 1 in particular, will become a strong competitor of the current

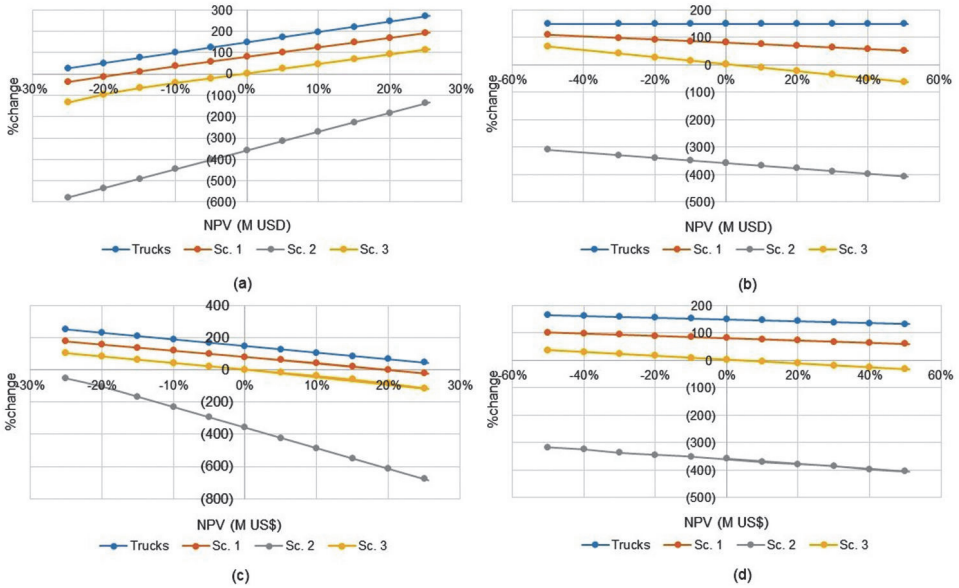

Figure 3. The impact of different parameter variations on NPV of the Subject mine: (a) coal price, (b) CAPEX, (c) OPEX, (d) distance.

trucking system given a 40% decrease or more of the capital expenditures. The variation of the capital costs affects the NPV of Scenario 2 as well, yet not enough to result in a positive value.

The variation of operational costs seems to have influenced the NPV of Scenario 2 even more (Figure 3c), since the specific pipeline system has higher operating costs among other alternatives. Nevertheless, even a 25% decrease of OPEX seems not to be enough for Scenario 2 to result in a positive NPV. The other three transport systems show similar trend of NPV as the results of operational costs variations indicate in Figure 3c.

A notable limitation in this study is that the financial analysis did not include the analysis of fuel price and power supply cost variation effect in the overall operating costs. The fuel price and power supply costs are two variables that significantly differentiate between the trucks system and slurry pipeline system. Therefore, as truck haulage is highly influenced by fuel costs, it might be necessary to include the fuel price analysis for a more detailed study.

4.2.3. Transportation distance

As already mentioned, the transportation distance is an important factor; pipe installation and replacement costs depend on the pipeline length. The relationship between distance changes and the calculated NPVs is shown in Figure 3d. In all scenarios, the NPV has a linear relationship with the hauling distance variation. Reduction of transportation distance will cause an increase in NPV, but will not change the superiority of trucks system over the pipeline system. However, the NPV of Scenario 2 is still negative even with a 50% distance reduction. What is worth mentioning in this last diagram is that the changing range of the values with respect to distance is not as high as with respect to the previous parameters. Concerning the trucks system, this can be attributed to the low labour cost that has already been discussed in other sections of this study and to the fact that a fuel price analysis for the trucks has not been included in this study. When it comes to the pipeline systems, the distance is not playing such a significant role as the CAPEX and OPEX to respectively build and operate such systems.

4.2.4. Recovery and price of liquid carrier

Scenarios 2 and 3, which use special liquid or mixture to transport the coal, are highly dependent on the recovery rate of the liquid. This is depicted in Figure 4. The assumed recovery rate of the liquid for pipeline Scenarios 2 and 3 is 95%. The 5% loss in each cycle significantly affected the operational cost of Scenario 2 in particular, whose liquids need to be purchased. The NPV of Scenario 2 will

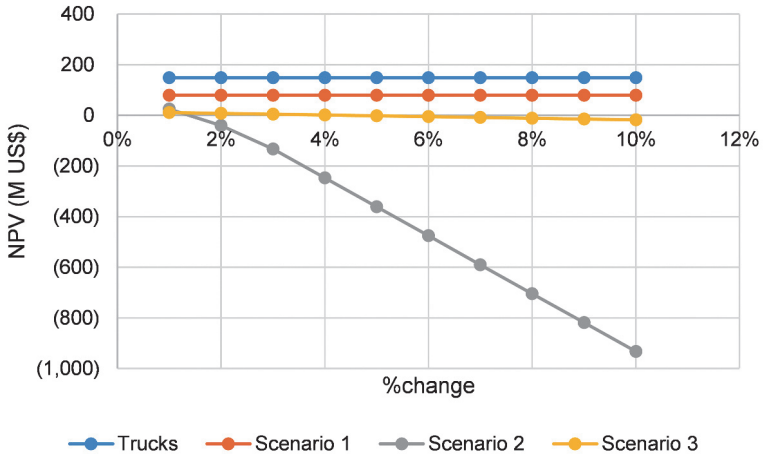

Figure 4. The impact of liquid losses variation to pipeline NPV.

increase dramatically and even become positive under very ideal conditions where 99% of the liquid is recoverable. Meanwhile, the variation of liquid losses only slightly affects the NPV of Scenario 3.

4.3. Wear of pipe

One of the biggest expenditures in pipeline financial estimation comes from the pipeline replacement due to pipe wear. If the pipe lifetime can be longer than 8 months, as assumed, then there is a chance to increase the economic attractiveness of slurry pipeline. Table 8 illustrates the impact of pipe life variation on NPV for all slurry pipeline scenarios. In this pipeline lifetime variation, only coal pipeline is considered and other pipeline systems such as pipeline to transport liquid, pumps, and all other parameters are in base case assumptions.

In addition, Table 8 shows that if the wear of pipeline can be reduced, which means a longer pipeline lifetime, the NPV of slurry pipeline will increase quite significantly especially for Scenarios 2 and 3 which utilise bigger pipe size.

4.4. Environmental analysis

Slurry pipeline systems offer better environmental performance in terms of air emissions (CO_2, NO_x), less dust and noise emissions compared to trucks systems. Coal transportation affects communities through which coal passes. Especially trucks hauling coal have the potential to damage roads and cause deaths or injuries in accidents. On the other hand, coal trains crossing local roads temporarily block those roads, adding traffic congestion and potentially delaying or degrading responses by police, fire, and other emergency responders and temporarily cutting off some residents from emergency services [4].

The pipeline transportation process also requires less energy due to less machinery required. However, there are some environmental aspects that should be assessed for slurry pipeline systems.

In hydro-transportation, utilisation of water can be a major issue that affects the whole operation. Water issue can be related to river water flow [30] or oppositions from society due to scarcity and water-protection-related problem [40]. Therefore, it is necessary to have a complete environmental impact assessment to identify risk and the required protection plans. To minimise water consumption, recirculating the liquid carrier to the mine is one of the significant effort that can be explored. Since it is not possible to produce zero waste of the slurry system, the waste produced at the end of the pipeline should be placed in a designated place with good liners or protection to avoid spoilage or leakage to the nearby environment. The waste water should also be treated before being discharged to the natural environment.

Other environmental impacts might be generated during the construction of pipeline system due to trenching and land clearing which will affect the natural vegetation [11]. Therefore, proper planning is necessary to minimise land opening that is also followed by revegetation plans and actions.

Table 8. Variation of pipeline lifetime to NPV of the pipeline scenarios.

Pipeline lifetime	NPV (US$)		
	Scenario 1	Scenario 2	Scenario 3
8 months	81,108,604	(357,685,503)	3,030,843
12 months	86,486,873	(348,846,043)	14,713,488
18 months	91,942,642	(339,879,208)	26,564,478
24 months	94,976,321	(334,893,201)	33,154,220

4.5. Social analysis

Part of what has just been discussed in the previous paragraph could be included in the social impact analysis as well. However, this section focuses on the social acceptance of an alternative coal transportation system in the context of job creations or losses.

The pipeline system evaluated in this study is projected to cover only 4.2 million tonnes of coal transportation per year which means it will only be used to support the increase of mining production. This should not generate any social issues with regard to job losses of current hauling contractors. However, it might be possible that in a later more detailed study, the slurry pipeline might become an attractive option to substitute trucks in coal transportation.

Currently, the Subject mine employs local contractors to provide coal hauling service as part of corporate social responsibility initiatives. This is one of the Subject mine's way to get engaged with surrounding community and hold the society trust by creating job and business opportunities. Therefore, it will not be a decent plan to completely replace the trucks system with slurry pipeline despite how beneficial it is. In the case where slurry pipeline can generate better financial profit and be considered as a good alternative of coal transportation method, Subject mine can use the slurry pipeline system as a subsidiary transportation system to avoid negative social impacts.

5. Conclusions

To conclude, some interesting comments are drawn. The initial scope of this study was to assess the technical and economic efficiency of coal slurry pipeline systems as alternative transportation methods for a specific coal mining operation in Kalimatan, Indonesia and compare them to the already established transportation system of trucks. A series of parameters have been discussed and specific boundary conditions were applied in the developed model that were addressed by the owner company. Three different slurry pipeline systems were tested and their technical and economic results, as well as environmental and social aspects, were evaluated against the respective outcomes of the truck transportation system. Given that this study is at a preliminary stage, a sensitivity analysis was also conducted.

From the point of view of the technical analysis, transporting coal via slurry pipelines has proved to be feasible with three options of liquid carriers. A slurry system that uses water as carrier will occur as turbulent flow; thus, a high particle degradation rate might occur at 30–40%. A more stable flow can be achieved if the liquid carrier has high density and high viscosity values; accordingly, in this study the assumed carrier was a liquid with physical properties like glycerine and a CSS-CWS mixture.

Despite the technical efficiency, the financial analysis for all three scenarios of slurry pipeline systems concluded that none of them can generate a higher value than the current trucks method. This is because the truck system has developed infrastructures and equipment while any of the proposed slurry pipeline systems requires high capital expenditures to set them up. The low trucking costs on one hand and the high pipeline operating costs on the other reinforce this concluding remark.

Nevertheless, the slurry pipeline system that uses water as carrier (Scenario 1) showed a competitive benefit in the case of capital expenditures being reduced. In one of the other systems (Scenario 2), a glycerine-like liquid is used as liquid carrier. A high operating cost due to the need to purchase the liquid is making this scenario unfeasible, but if palm oil waste is used as liquid carrier instead, the costs will decrease and this system might be feasible as well. Integration of coal transportation and palm oil waste recycling activities will be a good solution also for the protection of the environment. However, a later more detailed study will be necessary to evaluate its potential.

In any case and when one of the proposed slurry pipeline systems becomes a worthy alternative of coal transportation with trucks, a risk assessment needs to be conducted to evaluate all possible environmental and social impacts that might be generated. For water protection acts, recirculating

a liquid carrier back to the starting point and a proper waste water treatment at the end of the pipeline will be necessary. Furthermore, to avoid social arguments, any of the slurry pipeline systems developed should be applied as a subsidiary mode of the current truck haulage.

Overall, this study has investigated a topic that has never been discussed before. The comparison of inland coal transportation modes in a land with special boundary conditions proved to be challenging and despite the fact that the proposed coal slurry pipeline systems are not as economically efficient as the current truck system, a lot of critical evaluation parameters were determined and a robust financial model was developed, in which more precise data can be inserted to produce a better outcome and lead into more secure investment decisions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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List of Publications

Monei, N., Hitch, M., Heim, J., Heilmeier, H., Wiche, O. (2022). Effect of substrate properties and phosphorus supply on facilitating the uptake of rare earth elements (REE) in mixed culture cropping systems of *Hordeum vulgare*, *Lupinus albus* and *Lupinus angustifolius*. *Environ Sci Pollut* Res, 29, 57172–57189. doi: 10.1007/s11356-022-19775-x.

Monei, N. L., Puthiya Veetil, S. K., Gao, J., & Hitch, M. (2020). Selective removal of selenium by phytoremediation from post/mining coal wastes: practicality and implications. *International Journal of Mining, Reclamation and Environment*, *35*(1), 69–77. doi:10.1080/17480930.2020.1801118.

Wiche, O., Dittrich, C., Pourret, O., **Monei, N.**, Heim, J., & Lambers, H. (2023). Relationships between carboxylate-based nutrient-acquisition strategies, phosphorus-nutritional status and rare earth element accumulation in plants. *Plant and Soil*, 1–22. doi: 10.1007/s11104-023-06049-9.

Okoroafor, P. U., Mann, L., Amin Ngu, K., Zaffar, N., **Monei, N. L.**, Boldt, C., Reitz, T., et al. (2022). Impact of Soil Inoculation with *Bacillus amyloliquefaciens* FZB42 on the Phytoaccumulation of Germanium, Rare Earth Elements, and Potentially Toxic Elements. *Plants*, *11*(3), 341. MDPI AG. doi: 10.3390/plants11030341.

Jati, H. A., **Monei, N.**, Barakos, G., Tost, M., & Hitch, M. (2021). Coal slurry pipelines: a coal transportation method in Kalimantan, Indonesia. *International Journal of Mining, Reclamation and Environment*, *35*(9), 638–655.

Elulookirjeldus

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Teenistuskäik

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Välisuuringud

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Konverentsid ja seminarid

November 2022	XIV scientific conference of the Faculty of Science, Tallinn University of Technology, Estimaa
Mai 2022	"EGU General Assembly 2022, Biopathways of soil pollutants in soil-plant systems across scales - mechanistic understanding, risk assessment, and remediation approaches", Viin, Austria
Märts 2022	Circular Economy Sustainable Competencies for Youth, Erasmus+, Tartu, Estimaa
September 2021	"3rd Global Engineering Symposium" (Online)

September 2021	Raw Materials University Days 2021, Riia Tehnikaülikool, Läti
Aprill 2021	"EGU General Assembly 2021, Soil-Plant System on the polluted ecosystems recovery: Advances in bioremediation and biomining research", Vienna, Austria
Oktoober 2020	"EcoMining: Development of Integrated PhD Program for Sustainable Mining & Environmental Activities", Dnipro Tehnikaülikool, Ukraina
Aprill 2020	"EGU, General Assembly, Soil System Sciences Panel: Soil Pollution and Reclamation session. Advances in bioremediation and biomining research: for plants, soil and the rhizosphere", Viin, Austria
Märts 2020	"2nd Global Engineering Symposium", Bakuu, Aserbaidžaan
August– Oktoober 2019	Doctoral Course for Circular Economy and Raw Material Competence for Sustainable Production, Tallinna Tehnikaülikool & TU Bergakademie Freibergi, Saksamaa
Mai 2019	1st Society of Mining Professors (SOMP) PhD Candidate Research Days, Tallinna Tehnikaülikool, Geoloogia Instiuut
Aprill–August 2019	Doctoral Course for Circular Economy Entrepreneurship in System Integrated Metal Processing, TU Delft, Delft, Holland
Aprill 2019	Globaalne kliimamodelleerimine & piirkondlik kliimamodelleerimine, Tallinna Tehnikaülikool, Geoloogia Instiuut

Juhendatud lõputööd

2021	Vera Benyr (MSC), "Relationship between silicon accumulation and uptake of essential and non-essential elements in plants with different nutrition strategies", Bergakademie Freibergi Tehnikaülikool, Saksamaa

Publikatsioonid

Monei, N., Hitch, M., Heim, J., Heilmeier, H., Wiche, O. (2022). Effect of substrate properties and phosphorus supply on facilitating the uptake of rare earth elements (REE) in mixed culture cropping systems of *Hordeum vulgare*, *Lupinus albus* and *Lupinus angustifolius*. *Environ Sci Pollut* Res, 29, 57172–57189. doi: 10.1007/s11356-022-19775-x.

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