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POTENTIALLY TOXIC ELEMENTS FROM PHOSPHATE FERTILIZERS TO CROPS: MOBILIZATION AND MITIGATION

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para obtenção do título de Doutor.

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ELEMENTOS POTENCIALMENTE TÓXICOS DE FERTILIZANTES FOSFATADOS PARA CULTURAS: MOBILIZAÇÃO E MITIGAÇÃO

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APROVADA em 26 de março de 2024.
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À minha família, dedico!

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“Condições de palácio tem qualquer terra
larga, mas onde estará o palácio se não o
fizerem ali?” (Fernando Pessoa)

RESUMO

O fósforo (P) é um dos elementos requeridos para o crescimento e desenvolvimento das plantas. Portanto, aportes adequados de P no solo são essenciais para a produção de alimentos. No entanto, rochas fosfáticas podem conter naturalmente diferentes níveis de elementos potencialmente tóxicos (EPTs), como arsênio (As), cádmio (Cd) e cromo (Cr). Dentre os três elementos selecionados, o Cd é o mais facilmente biodisponível para as plantas e, portanto, para a cadeia alimentar. Assim, este elemento tem se tornado um desafio para alcançar uma produção alimentar sustentável e segura a nível mundial, especialmente após a nova regulamentação da União Europeia que visa reduzir os limites de Cd em fertilizantes fosfatados (UE 2019/1009). O aumento do pH do solo é uma das estratégias eficazes reduzir a biodisponibilidade do Cd no solo, bem como a suplementação mineral com elementos antagonistas ao Cd, como o selênio (Se). Neste contexto, objetivou-se com este estudo teve determinar os teores semi totais de As, Cd e Cr em três fertilizantes fosfatados (MAP 1, MAP 2 e MAP 3) com teores contrastantes de EPTs. O estudo também investigou o acúmulo e os impactos desses elementos no solo e em culturas relevantes, conhecidas pela sua tolerância contrastante a estes metais(loid)es (batata, tabaco, arroz), cultivadas em solo tropical, com e sem calagem. Também foram investigados a interação Cd-Se e os ajustes metabólicos para minimizar os distúrbios fisiológicos da planta (*Arabidopsis* e alface) devido à absorção de Cd. Uma gama diversificada de técnicas sensíveis para determinação de oligoelementos foi usada para revelar o nível mais alto de Cd do MAP 3. O MAP 3 resultou nas maiores concentrações deste elemento na matriz e na solução do solo, bem como na parte aérea de plantas e na seiva do xilema das plantas de batata e tabaco, contrastando com os resultados para MAP 1. O alto teor de Cd no MAP 3 reduziu os níveis de Mn e Zn nas folhas das plantas de batata e tabaco e, ao mesmo tempo, induziu um aumento de enzimas antioxidantes e açúcares em plantas de arroz. A acidez no solo aumentou as concentrações de Cd no solo e nas plantas, diminuiu o crescimento das plantas, a produtividade e o teor de açúcares, e induziu ajustes metabólicos relacionados às enzimas antioxidantes e à prolina. Entre as culturas, o arroz apresentou menor translocação – transporte à longa distância - de Cd em comparação à batata e ao tabaco, e maior tolerância ao pH ácido. Em relação ao Se, este elemento foi eficiente em aumentar o crescimento radicular, melhorar o desempenho fotossintético, estimular compostos do metabolismo S/Se e aumentar significativamente o teor de S, Mo e Fe, drasticamente reduzido pelo estresse por Cd. Nossos resultados evidenciam o uso de fertilizantes com baixo teor de Cd para reduzir os riscos de contaminação do solo, assim como a importância do manejo do solo e suplementação mineral para otimizar o crescimento e desenvolvimento de plantas expostas a esses elementos.

Palavras-chave: calagem; fertilizantes fosfatados; fitoquelatina; nutrição vegetal; segurança alimentar; selênio.

ABSTRACT

Phosphorus (P) is one of the required elements for plant growth and development. So, adequate inputs of P in the soil are essential for food production. However, phosphate rock may naturally contain different levels of potentially toxic elements (PTEs), like arsenic (As), cadmium (Cd), and chromium (Cr). Among the three selected elements, Cd is the most easily bioavailable to the plants, and so food chain. So, this element has been a stepping-stone toward achieving sustainable and safe worldwide food production, especially after a new European Union regulation aiming for reduced limits of Cd in P fertilizers (EU 2019/1009). Soil pH increase is one of the effective strategies to cope better with Cd in soil, as well as mineral supplemental with antagonist elements to Cd, like selenium (Se). In this context, this study aimed to determine the As, Cd, and Cr levels in three P fertilizers (MAP1, MAP2, and MAP3) with contrasting PTEs levels. It also investigated the accumulation and impacts of these elements in the soil and relevant crops known for their contrasting tolerance to these metal(loid)s (potatoes, tobacco, rice) grown in tropical soil, with and without liming. It also investigated the Cd-Se interaction and metabolic adjustments to minimize the plant physiology (Arabidopsis and lettuce) disturbance due to Cd uptake. A diverse array of sensitive techniques for trace elements determination were used to reveal the highest level of Cd of MAP 3. MAP 3 loaded the highest amounts of this element to the soil matrix and solution, as well as to plant shoots and the potato and tobacco xylem sap, contrasting with results for MAP 1. The higher level of Cd in MAP 3 reduced Mn and Zn levels in plant shoots for potatoes and tobacco while also inducing an increase of antioxidant enzymes and sugars in rice. Soil acidity increased Cd concentrations in soil and plants decreased plant growth, yield, and sugar content, and induced metabolic adjustments related to antioxidant enzymes and proline. Among the studied crops, rice had a lower Cd translocation – root-to-shoot transport - compared with potato and tobacco and a higher tolerance to acidic pH. Regarding Se, this element was efficient in increasing root growth, improving photosynthetic performance, stimulating S/Se metabolism compounds, and significantly increasing the content of S, Mo, and Fe, drastically reduced by Cd stress. Our findings highlight the use of low Cd fertilizers to reduce soil contamination risks, as well as the relevance of soil management and mineral supplementation aiming optimize the growth and development of plants exposed to these elements.

Keywords: food safety; liming; phosphate fertilizers; phytochelatin; plant nutrition; selenium.

Impactos sociais, tecnológicos, econômicos e culturais

Esse trabalho representa um estudo relevante que objetivou correlacionar fertilizantes fosfatados e elementos potencialmente tóxicos (arsênio, cádmio e cromo) em três culturas diferentes, utilizando uma ampla gama de técnicas sensiveis para determinação de elementos-traço. Além disso, também objetivou investigar como o selenio – um elemento benéfico para as plantas e essencial para humanos – pode minimizar os impactos causados pelo cádmio no metabolismo das plantas. Os resultados desse trabalho demonstraram que fertilizantes fosfatados com alto teor de cádmio pode resultar em contaminação do ambiente e cadeia alimentar. O estudo tem, portanto, potencial para impactar medidas regulatorias e servir como referência no desenvolvimento de bandeiras verdes para fertilizantes, especialmente em cenários onde o acúmulo de cádmio representar risco potencial para a saúde do solo e humana. Dentre os elementos avaliados, o cádmio é é o mais facilmente biodisponível para as plantas e, portanto, para a cadeia alimentar. Assim, este elemento tem se tornado um desafio para alcançar uma produção alimentar sustentável e segura a nível mundial, especialmente após a nova regulamentação da União Europeia que visa reduzir os limites de Cd em fertilizantes fosfatados (UE 2019/1009). Os impactos sociais que podem ser alcançados com esse trabalho está relacionado com a saúde pública, segurança alimentar porque a presença de elementos tóxicos pode comprometer a segurança e qualidade dos alimentos, e com isso pode acarretar problemas de saúde na população em cenários mais críticos. Além disso, foi abordado a necessidade de discutir potenciais riscos associados ao uso de fertilizantes fosfatados com alto teor de cádmio e as práticas de mitigação necessárias. Os resultados do trabalho podem ainda contribuir para inovações tecnológicas para monitorar e gerenciar a dinâmica de elementos tóxicos nos solos agrícolas. Adicionalmente, o trabalho pode ainda estimular as discussões relacionadas às regulamentações sobre níveis de elementos tóxicos em produtos agrícolas e sobre práticas agrícolas mais seguras e sustentáveis. Como pontuado acima, o estudo se relaciona direta ou indiratemente com os Objetivos de Desenvolvimento Sustentável (ODS) da ONU, por exemplo: alcançar a segurança alimentar, promover agricultura sustentável, assegurar padrões de consumo e produção sustentáveis.

Social, technological, economic and cultural impacts

This study represents a significant investigation aimed at correlating phosphate fertilizers with potentially toxic elements (arsenic, cadmium, and chromium) in three different crops, utilizing a broad range of sensitive techniques for trace element determination. Additionally, it aimed to explore how selenium—a beneficial element for plants and essential for humans—can mitigate the impacts of cadmium on plant metabolism. The results demonstrated that phosphate fertilizers with high cadmium content can lead to environmental contamination and entry into the food chain. Therefore, the study may influence regulatory measures and serve as a reference for developing green labels for fertilizers, especially in scenarios where cadmium accumulation poses a potential risk to soil and human health. Among the evaluated elements, cadmium is the most bioavailable to plants and, consequently, to the food chain. Thus, cadmium has become a challenge for achieving sustainable and safe food production worldwide, especially after the new European Union regulation aiming to reduce Cd limits in phosphate fertilizers (EU 2019/1009). The social impacts that can be achieved with this study are related to public health and food security because the presence of toxic elements can compromise the safety and quality of food, potentially causing health problems in the population in more critical scenarios. Additionally, the study addressed the need to discuss the potential risks associated with the use of high-cadmium phosphate fertilizers and the necessary mitigation practices. The results can also contribute to technological innovations for monitoring and managing the dynamics of toxic elements in agricultural soils. Moreover, the study can stimulate discussions related to regulations on toxic element levels in agricultural products and the adoption of safer and more sustainable agricultural practices. As noted above, the study directly or indirectly relates to the United Nations Sustainable Development Goals (SDGs), such as achieving food security, promoting sustainable agriculture, and ensuring responsible consumption and production.

SUMMARY

FIRST PART

1. INTRODUCTION

Brazil stands as a four-top global leader in food production, and the third-largest phosphate-consuming country in the world (Statista, 2021). Elevated acidity and Al^{3+} availability often found in tropical soils, combined with limited nutrient availability contribute to phosphorus (P) fixation and requirements of adequate P inputs for maximizing the crop yield and thus, food production (Lopes; Guilherme, 2016).

Phosphate fertilizers commonly used on agricultural soils are triple superphosphate (TSP), single superphosphate (SSP), monoammonium, and diammonium phosphate (MAP, DAP), all derived from apatite present in phosphate rocks (Saueia; Mazzilli, 2006). Ammonium phosphates (MAP, DAP) are considered the best P sources for plant growth (Suleman *et al.*, 2022) and represent about 50% of the P_2O_5 used in Brazil (Ifa, 2021), a country that ranks first position in MAP imports (Fao, 2021).

Phosphate rock deposits naturally may contain different levels of trace elements, mainly cadmium (Cd) with a wide range of concentrations varying according to the geological origin (igneous or sedimentary) (Cheraghi; Lorestani; Merrikhpour, 2012; Saueia; Mazzilli, 2006). Igneous phosphate deposits are known to have lower concentrations of Cd (<1-4 mg kg^{-1}) and are usually found in abundance in South Africa and Russia, while sedimentary rocks, usually found abundantly in Morocco, Togo, Senegal, Idaho, and the USA have the highest Cd concentrations (<1-150 mg kg^{-1}) (McLaughlin *et al.*, 1996; Roberts, 2014). Additionally, most phosphate rocks are usually extracted by sedimentary deposits, and the trace elements may be increased as impurities during the chemical process of phosphate fertilizer manufacturing (Jayasumana *et al.*, 2015; Van Kauwenbergh, 2010).

Among the metal(loid)s listed by USEPA (1999) and THE WEINBERG GROUP THROUGH THE FERTILIZER INSTITUTE (2001) as the main contaminants found in agricultural fertilizers, arsenic (As), cadmium (Cd), and chromium (Cr) are found in significant levels in phosphate fertilizers (Nziguheba; Smolders, 2008). Amongst these three elements, As and Cd levels are of greater concern due to their high toxicity (Verbeeck et al., 2020). Several studies have been correlating P inputs with an accumulation of potentially toxic elements (PTEs) in agricultural soils (Bracher *et al.*, 2021; Cheraghi *et al.*, 2012; Hu *et al.*, 2024; Park *et al.*, 2021; Suciu *et al.*, 2022; Suleman *et al.*, 2022; Verbeeck *et al.*, 2020). In Brazilian soils, positive correlations were related between the concentration of these elements and phosphate fertilizers, with PTEs concentrations often directly linked to P concentration in fertilizers (Guilherme *et al*., 2020).

Introducing PTEs into the soil by P fertilizers may lead to soil and food chain contamination, which might pose risks to both animals and humans. Thus, these concentrations are regulated by several instruments, *e.g.*, the Normative Instruction (IN SDA) 27/06 (MAPA, 2006), for Brazil. For phosphate fertilizers, the maximum concentrations allowed by this normative in Brazil are 2, 4, and 40 mg kg^{-1} for each 1% of P_2O_5, for As, Cd, and Cr, respectively. However, the maximum concentration for the total fertilizer mass is 250 and 57 for As and Cd, respectively (Cr has no such regulation). At the global commerce level, the European Union proposed a new regulation that came into effect in July 2022 (EC, 2019). Regulation 2019/1009 proposed reducing Cd levels in phosphate fertilizers and banishing fertilizers with Cd concentration above the threshold of 60 mg kg^{-1} P_2O_5. Moreover, it was established an eligibility for low Cd labeling for fertilizers with Cd < 20 mg kg^{-1} P_2O_5.

In addition to causing soil contamination and the input of these toxic elements into the food chain, the interactions between these elements with P and other essential elements to plants (nutrients) both in the soil and in plant metabolism are diverse and may affect the uptake and translocation of nutrients resulting in changes in impaired physiological processes compromising crop development and yield (Ismael *et al*., 2019; Naem *et al*., 2019). Hence, investigating the presence of these toxic elements in agricultural phosphate fertilizers and their impacts on plant nutrition and physiology is paramount to ensuring the quality of agricultural inputs and the sustainability of agroecosystems.

Various agronomic and biological approaches have been proposed to mitigate the adverse effects of PTEs on soil and plants and prevent their bioaccumulation in the food chain, especially Cd, the most mobile and bioavailable metal in agricultural soils. Altering soil nutritional profiles is pivotal in reducing plant uptake of these elements, which is achievable through management practices like pH adjustment with limestone and P supplementation through P fertilization (Pierangeli *et al*., 2004, 2005, 2009). Indeed, phosphate can be used to immobilize cadmium in soils (Liao *et al*., 2013; Yan; Zhou; Liang, 2015), but as Cd and other PTEs are commonly found as contaminants in P fertilizers, liming arises as an effective strategy for minimizing higher Cd levels in soil (Zhao; Wang, 2020).

Mineral nutrition and supplementation are also used to mitigate Cd and other metal(loid)s toxicity in plants (Dos Santos *et al*., 2020; Oliveira *et al*., 2020). In this context, selenium (Se) has been extensively investigated due to its antioxidant beneficial effects on plants and essentiality for humans (Boldrin *et al.*, 2018). Previous studies have shown that Se can decrease Cd accumulation in grains and plant tissues, alleviate oxidative stress, increase photosynthesis, and improve growth to minimize the toxicity effects of Cd exposure (Alves *et al.*, 2020; Gao *et al.*, 2018; Li *et al.*, 2020; Riaz *et al.*, 2021).

Despite several shreds of evidence about the antioxidant role of Se during Cd stress, other beneficial mechanisms of selenium against abiotic stress still need to be clarified. Selenium shares the uptake and assimilation metabolism with sulfur (S), essential for plants (Anjum *et al.*, 2015). Some of the products from S metabolism have been correlated to Cd stress in plants, such as glutathione (GSH) and phytochelatin (PCs) (Gill *et al.*, 2012). So, we highlight the relevance of investigating if Se regulates S-compounds to minimize Cd toxicity in plants.

For this study, we selected some plant species known for their distinctive metal(loid)s tolerance and also based on biological and agricultural relevance to investigate PTEs from P fertilizers, the role of liming on the availability of these elements, and finally discuss mitigation strategies of Cd, the most bioavailable PTEs for plants.

So, in Manuscript one, we first assessed As, Cd, and Cr levels in three different MAP fertilizers, from three different geographic origins, to quantify the uptake and translocation of these elements in two soil pH ranges for economically relevant crops: potato, tobacco, and rice. Vegetables and cereals are major sources of PTEs in the food chain (Corguinha *et al.*, 2015). Potato and rice are staple foods, which represent relevant sources of intake of PTEs, specifically Cd (Corguinha *et al.*, 2015; Duan *et al.*, 2017; Mengist *et al.*, 2017; Zhao; Wang, 2020; Ye *et al.*, 2020). Besides, tobacco is also an important economic cash crop and is a potential source of Cd intake for humans (Mei *et al.*, 2022).

After evaluating the PTEs levels in fertilizers and crops, in Manuscript 2, we focused on the physiological impacts resulting from PTEs availability for plants under contrasting soil acidity conditions. Plant primary metabolism was evaluated through the growth, yield, photosynthesis-correlated parameters, antioxidant system, macromolecule content, as soluble sugars, and nutritional status. Finally, in the third Manuscript, we selected Cd, our worst-case scenario, to investigate a mitigation approach using Se as a beneficial element. For this purpose, we used lettuce and *Arabidopsis* as toxicity models to evaluate phenotype, photosynthesis, nutritional status, and S-compounds as non-protein thiols and enzymes of S assimilation.

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SECOND PART

Manuscript 1: Arsenic, cadmium, and chromium concentrations in contrasting phosphate fertilizers and their bioaccumulation by crops: towards a green label?

Manuscript under revision in Environmental Research (Impact factor: 8.3)

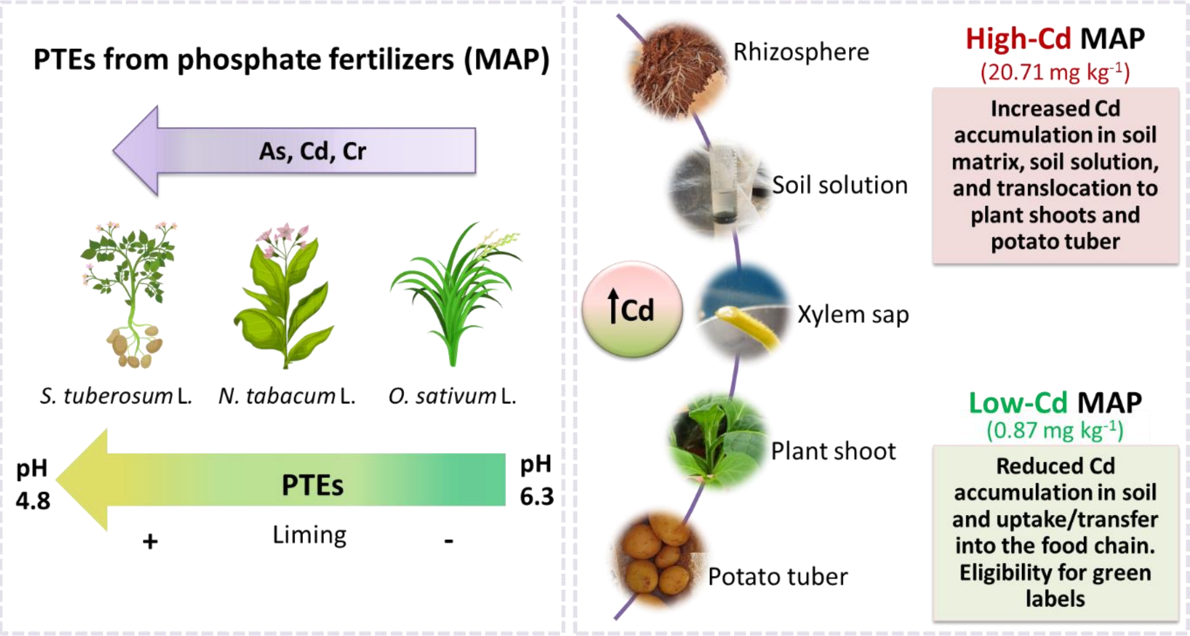
Highlights

- Phosphate fertilizers may represent sources of potentially toxic elements for agriculture.

- High-Cd P fertilizers increase Cd in xylem sap and its transfer from soil to plant tissues.

- Using low-Cd P fertilizers reduces Cd accumulation/transfer into the food chain.

- Potato crops have a high potential to accumulate Cd from P fertilizers.



Increased Cd accumulation in soil matrix, soil solution, and translocation to plant shoots and potato tuber

Reduced Cd accumulation in soil and uptake/transfer into the food chain. Eligibility for green labels

Abstract

Potentially toxic elements such as arsenic (As), cadmium (Cd), and chromium (Cr) are severely regulated in fertilizers and deserve continuous investigation. Phosphate-derived Cd has been a stepping-stone toward achieving sustainable and safe worldwide food production, especially after a new regulation aiming for reduced limits of Cd in P fertilizers (EU 2019/1009). Three pot experiments were conducted to assess the variability of As, Cd, and Cr concentrations - with a particular focus on Cd - from monoammonium phosphates (MAP 1, MAP 2, and MAP 3 from different geographic origins) and their accumulation in limed and unlimed soils, and contrasting crops, representing staple food and significant sources of these elements for humans (*i.e.*, potato, tobacco, and rice). A diverse array of sensitive techniques for trace elements determination were used to reveal the highest level of Cd of MAP 3 (20.71 mg kg^{-1} MAP), which loaded the highest amounts of this element to the soil matrix and solution, plant shoots, and xylem sap, contrasting with results for MAP 1 (0.87 mg kg^{-1} MAP), which has almost ten times less Cd than that required for low-Cd labeling of P fertilizers (≤ 20 mg Cd kg^{-1} P_2O_5). MAP 3 also had the highest Cr concentration (139.3 mg kg^{-1} MAP). Among crops, rice accumulated 16-fold less Cd than potato plants. Liming decreased Cd in tobacco and potato shoots up to 35%. Moreover, reductions of about 20% were also observed for Cd accumulation in tubers and sap. Conversely, Cd from MAP 3 was always much more accumulated in soil solution, achieving up to 20 µg L^{-1}, while values <5 µg L^{-1} (*i.e.*, a groundwater limit) were obtained from MAP 1. Our findings may be used as reference in developing green labels for fertilizers in scenarios where cadmium accumulation represents a potential risk for soil and human health.

Keywords: cadmium translocation, food safety, P fertilizers, potentially toxic elements, soil contamination.

1. Introduction

Phosphorus (P) is a required element for the plant life cycle and participates in many metabolic pathways and physiological processes (Malhotra et al., 2018). Plants uptake P from soil solution, so adequate inputs of P in the soil are essential for food production. Consequently, P is the basis of many fertilizers that provide plant nutrients. In 2021, 29,705.7 thousand metric tons of P fertilizers were used by the four top food-producing countries - China, India, the USA, and Brazil, with Brazil being the third largest phosphate-consuming country in the world (STATISTA, 2021).

Phosphate rocks are the major sources of P fertilizers worldwide. However, phosphate rock deposits of different origins naturally contain distinctive levels of potentially toxic elements (PTEs), and these elements can also be introduced as impurities from acids during the manufacturing process (Gupta et al., 2014). Thus, P fertilizers are a potential diffuse source for PTEs due to containing significant levels of these elements such as cadmium (Cd), chromium (Cr), and arsenic (As) (Nziguheba; Smolders, 2008). Recurrently application of P fertilizers has led to the accumulation of these elements in soil, with Cd being the most recurrent in agricultural soils around the world (Bracher et al., 2021; Cheraghi et al., 2012; Hu et al., 2024; Park et al., 2021; Suciu et al., 2022; Suleman et al., 2022; Verbeeck et al., 2020).

Among the three selected elements (As, Cd, and Cr), As and Cd contents in P fertilizers are of more significant concern due to their toxicity (Verbeeck et al., 2020). Phosphate and arsenate are chemically close, so, these elements compete for sorption sites in the soil, with As being released in the soil after P fertilizer application. While As also competes with P for binding sites in plant rhizosphere, both As and Cd, might also replace P in soil-root transport (Gupta et al., 2014). Thus, soil application of P fertilizers containing these elements can increase As and Cd mobility and bioavailability in soil-plant systems. Cadmium presence in phosphate fertilizers can vary widely according to their geological origin (igneous or sedimentary) and geographic location (Saueia and Mazzilli, 2006).

Cadmium contamination from fertilizers is relevant in some countries, such as China, where more than 50% of the environmental Cd comes from phosphate fertilizers (Tirado and Allsop, 2012). Cadmium as an impurity in fertilizers might pose risks to the environment, food safety, and animal and human health. Thus, the level of these elements must be avoided or constrained. In agreement with this, on June 5, 2019, the European Parliament and EU Council released the EU Fertilizing Product Regulation 2019/1009 (EC, 2019) that came into effect in 2022.

This EU regulation proposed reducing Cd levels in P fertilizers and decided to ban inorganic fertilizers with Cd content above the threshold of 60 mg kg^{-1} P_2O_5 since July 2022. In addition, fertilizers with Cd levels below 20 mg kg^{-1} P_2O_5 could be eligible for labeling indicating low Cd or similar, which can be helpful to improve market communications, mediate purchase decisions, and help protect the environment and human health.

After the publication of the EU regulation of Cd in fertilizers, the number of studies of "cadmium" and "fertilizer" as search topics increased significantly in the Web of Science database (https://www.webofscience.com/wos/woscc/summary/718ac877-3801-4b7d-a34f-e2336b1ab9d5-be8ff78b/relevance/1). Indeed, several studies can be found in the literature about toxic elements in fertilizers in last years (Bracher et al., 2021; Marini et al., 2020; Park et al., 2021; Ulrich, 2019; Verbeeck et al., 2020). Most studies have focused on Cd, other phosphate sources, or European soils. So, there are still gaps concerning the answer to questions like: What are the potential health risks associated with consuming crops grown in tropical soils using ammonium phosphate fertilizers containing toxic elements like cadmium, arsenic, and chromium? How can these potential risks be mitigated?

Among the top food-producing countries, Brazil ranks first in monoammonium phosphate – MAP imports, three times more than the second position (FAO, 2021). Ammonium phosphate fertilizers (especially monoammonium phosphate – MAP) represent about 50% of P_2O_5 used in Brazil (IFA, 2021). A comparative study with several P sources showed that ammonium phosphates (MAP and DAP) were better for plant growth than other P sources under tropical conditions. However, the maximum Cd in soil was also observed with MAP, compared with another eleven P sources (Suleman et al., 2022). This is also affected by soil pH, which influences P and Cd availability in soil. Indeed, one of the more relevant inorganic amendments on Cd availability is liming (Hussain et al., 2021; Ullah et al., 2021).

Given the importance of P fertilizers, especially MAP, and their role in food production, as well as the relevance of addressing the safety of such inputs concerning their content of PTEs, this study aimed to investigate the availability of arsenic, cadmium, and chromium in the soil and in relevant crops (potatoes, tobacco, and rice) fertilized with different P sources of this same basis (*i.e.*, MAPs of different geological origin) in a tropical soil with and without lime application. We tested the hypothesis that P fertilizers with low levels of As, Cd, and Cr could immobilize these elements in soils, reducing their bioavailability, bioaccumulation, and phytotoxicity, having a minimum environmental impact and allowing safe plant production for human health. With that, we hope to contribute to a better understanding of the importance of PTEs in phosphate fertilizers and their potential impact on soil contamination and food safety, which is especially relevant in tropical agroecosystems that use high rates of P fertilization.

2. Materials and Methods

2.1. The study site and plant materials

The experiments were conducted in 2021 and 2022 at the Department of Soil Science, Federal University of Lavras, Lavras, Minas Gerais, Brazil. To evaluate the uptake, transport, and accumulation of PTEs from phosphate fertilizers, we used three monoammonium phosphate fertilizers (MAP) with contrasting values for Cd content, and three experiments were set up with three crops of agronomic interest: potato (*Solanum tuberosum* L. cv Asterix), tobacco (*Solanum tabacum* L. Flue-Cured Virginia – cv CSC4704), and rice (*Oryza sativa* L. cv CMG 1590).

2.2. Elemental analyses in soil and fertilizers before experiments

The soil collected for experiments was taken from coordinates 18°58’17” S and 48°12’30” W at an altitude of 854 m (Uberlandia, Minas Gerais, Brazil), which corresponds to a representative tropical soil (Cerrado biome) with a fraction of clay content <35%. Soil samples were dried in the oven at 65°C for 72 hours, grounded, passed through a 2-mm nylon sieve, and analyzed for physicochemical properties following Brazilian standard procedures (Teixeira et al., 2017) (**Table 1**).

Table 1. Physicochemical properties measured of a Red-Yellow Latosol before liming and fertilizer treatment from 0-20 cm depth[1].

Clay (%)	32
Silt (%)	6
Sand (%)	62
pH (water)	5.10±0.4
Organic matter (%)	1.01±0.06
P (mg dm^{-3})	0.00
P-Rem (mg L^{-1})	14.5±0.43
K (mg dm^{-3})	39.45±0.4

Zn (mg dm^{-3})	1.03±0.06
Fe (mg dm^{-3})	73.83±3.89
Mn (mg dm^{-3})	8.83±0.21
Cu (mg dm^{-3})	1.57±0.08
B (mg dm^{-3})	0.28±0.01
S (mg dm^{-3})	4.07±0.35
Ca (cmol$_c$ dm^{-3})	0.19±0.12
Mg (cmol$_c$ dm^{-3})	0.16±0.03
Al (cmol$_c$ dm^{-3})	0.0
Potential acidity (cmol$_c$ dm^{-3})	2.63±0.11
CEC (cmol$_c$ dm^{-3})	3.08±0.06
BS (%)	14.40±4.76
m (%)	0.0

[1] Determined according to EMBRAPA (1997) and Teixeira et al. (2017). CEC: cation exchange capacity at pH 7.0; BS: base saturation; and m: Al saturation. Potential acidity: ($H^+ + Al^{3+}$). Textural analyses (g kg^{-1}): coarse sand = 231; fine sand = 485; silt = 45; clay = 239.

In the primary analysis, the soil pH was determined in water with a pH Meter using a soil: solution ratio of 1:2.5 after shaking and 1-hour rest. Base saturation [BS = $Ca^{2+} + Mg^{2+} + K+/T * 100$)] and cation exchange capacity [CEC = $Ca^{2+} + Mg^{2+} + K^+ + (H^+ + Al^{3+})$] (EMBRAPA, 1997) were considered in calculations of soil correction for establishing two treatments considering two contrasting soil pH: soil without liming (5.1, native soil pH), and soil with liming to reach a base saturation ~80% (pH 6.3), before cultivation. For this, a mixture of calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$) (3:1) with a relative neutralizing value (RNV) of 105% was used in a proportion of 1 g for 1 kg. Soil pH was monitored during incubation at 70% of field capacity (**Table S1**).

The pseudo total content of PTEs in soil and P fertilizers was determined following the USEPA 3051A method (United States Environmental Protection Agency – USEPA, 2007). First, all samples were air-dried, homogenized, ground, and 150-µm sieved before the acid digestion. Next, soil, fertilizers, and plant samples (0.250 g) were added to Teflon vessels with 5 mL of nitric acid (HNO_3), which were filled with ultrapure water (Milli-Q system) to 20 mL. This mixture was digested for 40 min in a microwave oven (MARS-5 ®, CEM), and after digestion, the solutions were cooled, filtered through Whatman no. 40 filter paper, and received a volume of 50 mL of Milli-Q water. Standard reference materials and blanks were carried out equally. Chromium was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), whereas As and Cd concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Standard reference materials (SRM) from the National Institute of Standards & Technology (NIST) – SRM 2709a San Joaquin Soil (Gaithersburg, MD, USA) – were used to check the accuracy and quality control of soil analytical results. Likewise, the accuracy of fertilizer analyses was checked using SRMs from the Institute for Reference Materials and Measurements – BCR 032 Moroccan Phosphate Rock (Geel, Belgium).

2.3. Growth conditions

Potato seeds were planted (4 seeds per pot) on June 22, 2021, in pots filled with 7 kg of soil and the P treatments. One week after planting, the first seedlings started to emerge. After three weeks (*i.e.*, on July 13, 2021), thinning was made to maintain two plants per pot. The treatments in the pots followed as described below: fertilization with 0.6 g P kg^{-1} of soil for each P source in treatments MAP 1, MAP 2, and MAP 3, in which MAP was mixed with the soil before planting. Nitrogen, potassium, and other nutrients were supplied and split four times via nutrient solutions based on the doses proposed by Malavolta (1980) and on data published in the literature of pot experiments with potatoes (De Oliveira et al., 2019), as follows: 380 mg kg^{-1} N; 400 mg kg^{-1} K; 200 mg kg^{-1} Ca; 50 mg kg^{-1} Mg; 50 mg kg^{-1} S; 0.5 mg kg^{-1} B; 1.5 mg kg^{-1} Cu; 2 mg kg^{-1} Mn; 5 mg kg^{-1} Mo; 5, mg kg^{-1} Zn; 5 mg kg^{-1} Fe.

The tobacco seeds were germinated in 5 kg soil pots in September 2021. The pot treatments were as follows: fertilization with 0.4 g P kg^{-1} of soil for each P source in treatments MAP 1, MAP 2, and MAP 3, in which MAP was mixed with the soil before planting. Nitrogen and potassium, as well as other nutrients, were supplied through nutrient solutions based on the doses proposed by Malavolta (1980) and in the literature concerning pot experiments with tobacco, as follows: 200 mg kg^{-1} N; 50 mg kg^{-1} K; 200 mg kg^{-1} Ca; 50 mg kg^{-1} Mg; 50 mg kg^{-1} S; 0.5 mg kg^{-1} B; 1.5 mg kg^{-1} Cu; 2 mg kg^{-1} Mn; 5 mg kg^{-1} Mo; 5, mg kg^{-1} Zn; 5 mg kg^{-1} Fe. For N and S, the recommended dose was divided.

To set up the rice experiment, pots were filled with 5 kg of soil, and the treatments were outlined as previously described. Twenty rice seeds were sown (*Oryza sativa* L. cv CMG 1590) per pot on November 10, 2021. After four weeks, the thinning was made to maintain three plants per pot. The treatments with MAP involved the fertilization with 0.25 g

P kg^{-1} of soil for each P source in treatments MAP 1, MAP 2, and MAP 3, in which MAP was mixed with the soil before planting. Basic fertilization was done with nutrient solutions following the doses proposed by Malavolta (1980) as well as by data from the literature concerning pot experiments with rice, which consisted of 80 mg kg^{-1} N, 70 mg kg^{-1} K, 60 mg kg^{-1} S, 200 mg kg^{-1} Ca, and 50 mg kg^{-1} Mg at planting, whereas fertilization with micronutrients consisted in the application of 3.6 mg kg^{-1} Mn, 1.5 mg kg^{-1} Cu, 5 mg kg^{-1} Zn, 0.5 mg kg^{-1} B, and 0.15 mg kg^{-1} Mo. During the growing period, rice plants will receive top-dressed N (450 mg kg^{-1}) and K (350 mg kg^{-1}), split into eight applications (Boldrin et al., 2013).

For all experiments, Mg and Ca were supplied only in pots without liming at a rate equivalent to those in pots receiving previous treatment with liming. The Control treatment consisted of soil without fertilizers.

2.4. Evaluation of Cd in soil solution and xylem sap

Micro rhizon samplers were used to collect the soil solutions in pots with potato plants, to analyze their Cd content further. Initially, a micro rhizon sampler (10 cm long, 2.5 mm diameter) with porous plastic (PES) and standard 0.15-micron pores was installed in each pot after sowing the seed potatoes. After installation, the system was assembled, containing a plastic hose coupled to a syringe, which was subjected to pressure to allow the solution to be collected. The collections were made four times: at 40, 58, 70, and 88 days after planting (DAP). The samples were stored at -80°C until later use.

To evaluate Cd concentration in the xylem at 77 days after planting, a stem from one potato plant selected in each pot was used to collect the sap. The collections were carried out before sunrise (05:00 AM) by cutting the stem, followed by exudate collection with a 10-µL micropipette. A similar approach was used for tobacco plants 135 days after sowing. All collected exudates were stored in liquid nitrogen and then frozen at -80°C until later use. The samples of soil solution and xylem sap were diluted, and Cd determination was carried out using graphite furnace atomic absorption spectrometry (GFAAS).

2.5. Elemental contents in soil and plants after experiments

To quantify PTEs contents, the soil samples from each replicate were homogenized before sampling for analysis, and then processing was followed as described before using USEPA Method 3051A (Environmental Protection Agency, 1998), which assesses pseudo-total contents in soils. The plant samples were washed in running water and distilled water before analysis. Leaves, tubers, and grains were placed separately in an oven at 65°C for 72 hours for drying until constant weight, and then processing was followed as described before (USEPA, 2007). Certified reference materials were used to check the accuracy of the quantification analysis for fertilizers (BCR 032), soil (San Joaquin Soil, SRM 2709a), and plants (Tomato Leaves, NIST 1573a). The analytical validation was performed considering the detection limit (DL), and quantification limit (LQ). The final values were obtained considering the sample dilution factor and sample weight. The same procedure was made for all experiments.

2.6. Statistical analysis

All samples were subjected to statistical evaluation on the software R 4.2.3 ($p < 0.05$) (R Development Core Team, 2018). The Shapiro-Wilk test was used to test the normality and the homogeneity of variance for data, and log transformations were conducted when necessary. Since the assumptions were not met even after data conversion, the non-parametric Wilcox and Kruskal-Wallis tests for multiple group comparison were used, followed by Dunn's test.

3. Results and Discussion

This study determined the As, Cd, and Cr pseudo-total concentrations in fertilizers, soil, and plants. Recoveries for these elements varied from 31 to 79% in soil and 34 to 107% in plants, with the higher % values found for Cd, validating the results (**Table S2**). Such discrepancies can be explained by the fact that USEPA 3051A is a method that evaluates pseudo-total concentrations in soils. Irrespectively of that, except for As (16.35 mg kg^{-1}), the average natural background content for Cd (0.25 mg kg^{-1}) and Cr (42.24 mg kg^{-1}) (**Fig. 1**) in the soil was smaller than the levels allowed by the Brazilian legislation, as follows: 15mg kg^{-1} for As, 1.3 mg kg^{-1} for Cd, and 75 mg kg^{-1} for Cr (CONAMA, 2009). Concerning arsenic, a similar value for As was found in natural soils from the State of Minas Gerais (17.8 mg kg^{-1}) (Caires, 2009), while values between 28 to 58 mg kg^{-1} were reported for Brazilian agricultural soils (De Menezes et al., 2020).

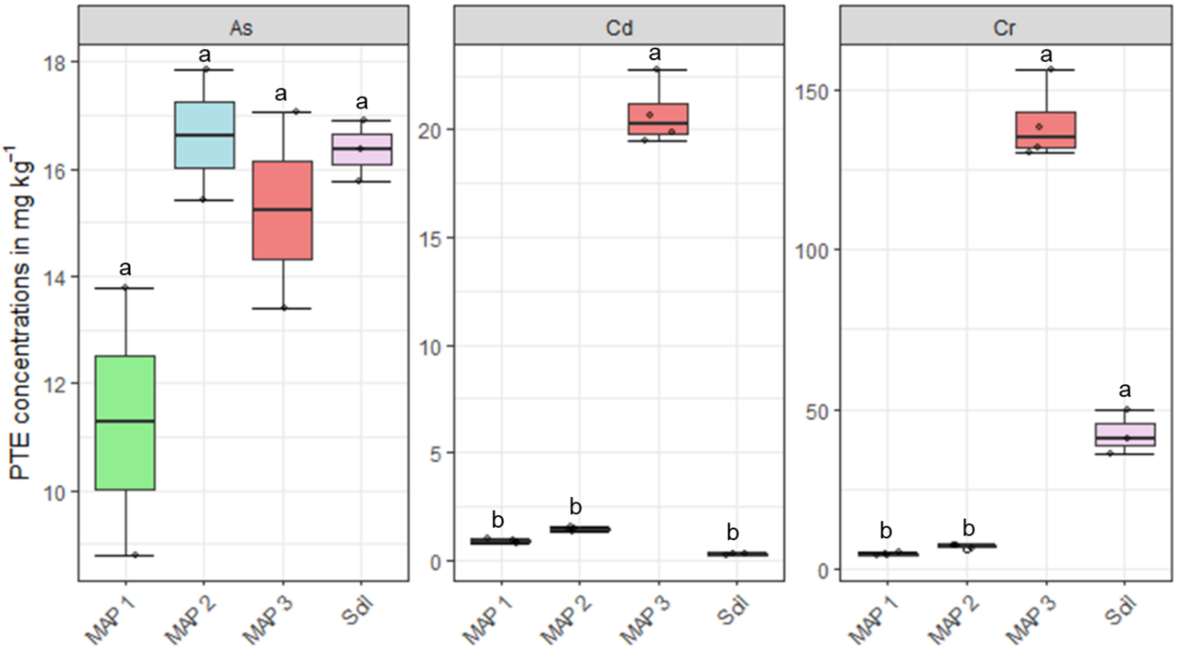


Figure 1. Pseudo-total arsenic (As), cadmium (Cd), and chromium (Cr) concentrations in P fertilizers and soil before the experiments. Data represents a distribution from four replications with a mean (black middle line). Different letters represent differences among samples at a probability of 5%, according to Dunn's test.

The soil used in this experiment, a Red-Yellow Latosol (Typic Dystrophic Red Latosol – Haplustox, according to the Soil Taxonomy), with medium texture, is a representative tropical soil of Brazil. After liming and at the end of the incubation period, the soil pH average was 4.82 for samples without lime and 6.30 for the samples with lime (**Table S1**). Tropical soils have low pH (acidic soils), stable iron and aluminum oxides, and low organic matter. These characteristics can interact with PTEs, depending on each element and soil type, through the process of adsorption, ion exchanges, precipitation, complexation, dissolution, and other chemical reactions (Fontes and Gomes, 2003; Guerra Sierra et al., 2021).

The averages of pseudo-total concentrations of As, Cd, and Cr found in the different fertilizers are shown also in Figure 1. The As concentrations of the evaluated samples were not significantly different ($p>0.05$). The highest Cd concentration (20.71 mg kg^{-1} MAP) was observed in MAP 3 ($p<0.05$) and is 14-fold greater than the Cd concentration in MAP 2 (1.43

mg kg^{-1} MAP), and 24-fold higher than that of MAP 1 (0.87 mg kg^{-1} MAP). MAP 3 also had the highest Cr concentration (139.33 mg kg^{-1} MAP).

In our study, the values observed in MAP 3 for these elements are close to those reported for P fertilizers from sedimentary phosphate rocks, *i.e.*, those known for their higher Cd contents (Hu et al., 2024; Veerbeck et al., 2020). Phosphorus is a primary nutrient for plant growth and a determinant of agriculture production (Bindraban et al., 2020). Monoammonium phosphate (MAP) is one of the P sources most applied in tropical agriculture and is derived from apatite present in phosphatic rocks. The phosphatic rocks contain varied mineral deposits with different levels of trace elements, especially Cd, with concentration changing according to geological origin (igneous or sedimentary) and geographic localization (Saueia and Mazzili, 2006; Cheraghi et al., 2012). So, the MAPs used in this study are from different geologic deposits, and the differences observed are according to the literature.

Notably, many phosphate fertilizers are extracted from sedimentary rocks (Van Kauwenbergh, 2010). Because of that, it has been quite common to use P fertilizers with relevant concentrations of Cd and other PTEs. In Brazil, the maximum values allowed by normative 27/2006 for As, Cd, and Cr in P fertilizers are respectively 2, 4, and 40 mg kg^{-1} for each 1% of P_2O_5, or a maximum amount – in mg kg^{-1} – in the total mass of fertilizer of 57 for Cd and 250 for As, whichever is more restrictive (Brasil, 2006). These two guidelines must be carefully addressed since MAP fertilizers usually have 52% P_2O_5.

Worldwide, some strategies have been established to minimize the exposure to PTEs and their food-chain transfer, especially Cd. Switzerland pioneered the establishment of the Cd limit value of 21 mg kg^{-1} P_2O_5 for mineral fertilizers and 11 mg kg^{-1} for P recycled from waste (Ulrich, 2019). Later, the Australian government started using low Cd phosphate fertilizers and labels about Cd levels in these products (Mead, 2010). The European Union (EU) has been concerned about Cd in fertilizers for the last decades and keeps trying to reduce Cd exposure through food products (Marini et al., 2020; Ulrich, 2019).

In this context, the EU recently introduced a new regulation (effective from July 2022) with a stricter limit of 60 mg kg^{-1} P_2O_5 for Cd in phosphate fertilizers. The regulatory idea was to start with 60 and decrease until 20 mg kg^{-1} P_2O_5, based on slower Cd accumulation in 100 years compared to values > 20 mg kg^{-1} P_2O_5. However, there has yet to be a consensus among members about it (Ulrich, 2019). The section “PRODUCT-SPECIFIC LABELLING REQUIREMENTS” proposes a statement “Low cadmium content” or similar for phosphate fertilizer with Cd content equal to or lower than 20 mg kg^{-1} of P_2O_5 (EU 2019/1009). Considering the presence of 52% of P_2O_5 in MAP fertilizers, this is equivalent to 10.4 mg Cd kg^{-1} MAP. In our study, only MAP 1 (0.87 mg Cd kg^{-1}) and MAP 2 (1.43 mg Cd kg^{-1}) presented Cd content almost ten times less than that threshold, while a concentration 2-fold greater was observed in MAP 3 (20.71 mg kg^{-1}) **(Fig. 1**).

A survey in 2020 reported that the Cd mean for P fertilizers consumed in EU countries was 30 mg kg^{-1} P_2O_5, and approximately 10% of fertilizers were above the Cd limit of 60 mg kg^{-1} P_2O_5 (Verbeeck et al., 2020). Thus, cadmium levels in fertilizers are going to be a global commerce limitation, especially after the dissemination of the Cd levels in dark chocolate and EU limits to Cd in cacao products (Abt et al., 2018; 2020; European Commission et al., 2019; Vanderschueren et al., 2021, 2023). It is also important to note that the international agenda has been moving towards soil health and food safety. So, the discussion about Cd limits is far from over.

High amounts of P fertilizers are often needed to achieve high yields in tropical agroecosystems, mainly due to the high P-fixation capacity of oxidic soils (Withers et al. 2018), which negatively affects the availability of P for plants (Gama-Rodrigues et al., 2014). Furthermore, for crops with higher P requirements such as potatoes, Cd and other toxic elements coming from P fertilizers might accumulate in the soil and crops. Cadmium accumulation in significant crops (potatoes, soybean, wheat, rice, and corn) was investigated in Brazilian areas with long-term use of P fertilizers by Corguinha et al. (2012, 2015). The crop, genotypic variability, and area were variation factors, but the Cd content was generally under *Codex Alimentarius* guidelines. Although the Cd contents in edible parts of the crops found by the authors did not represent a risk to human health, using fertilizer with low PTEs concentration is a relevant step to minimize biomagnification, avoid risks to human health, and pursue food safety.

In this study, after crop growth, the pseudo-total As concentrations in the soil varied between 0.01–4.04 mg kg^{-1} and were not significantly different ($p>0.05$) among the treatments for all experiments (**Fig. 2, 3, and 4**). Otherwise, a pH effect on As concentration can be observed only for the potato experiment, with a mean of 2.1 mg kg^{-1} for soil without liming (pH 4.8) and 1.3 mg kg^{-1} for soil with liming (pH 6.3) (**Fig. 2**).

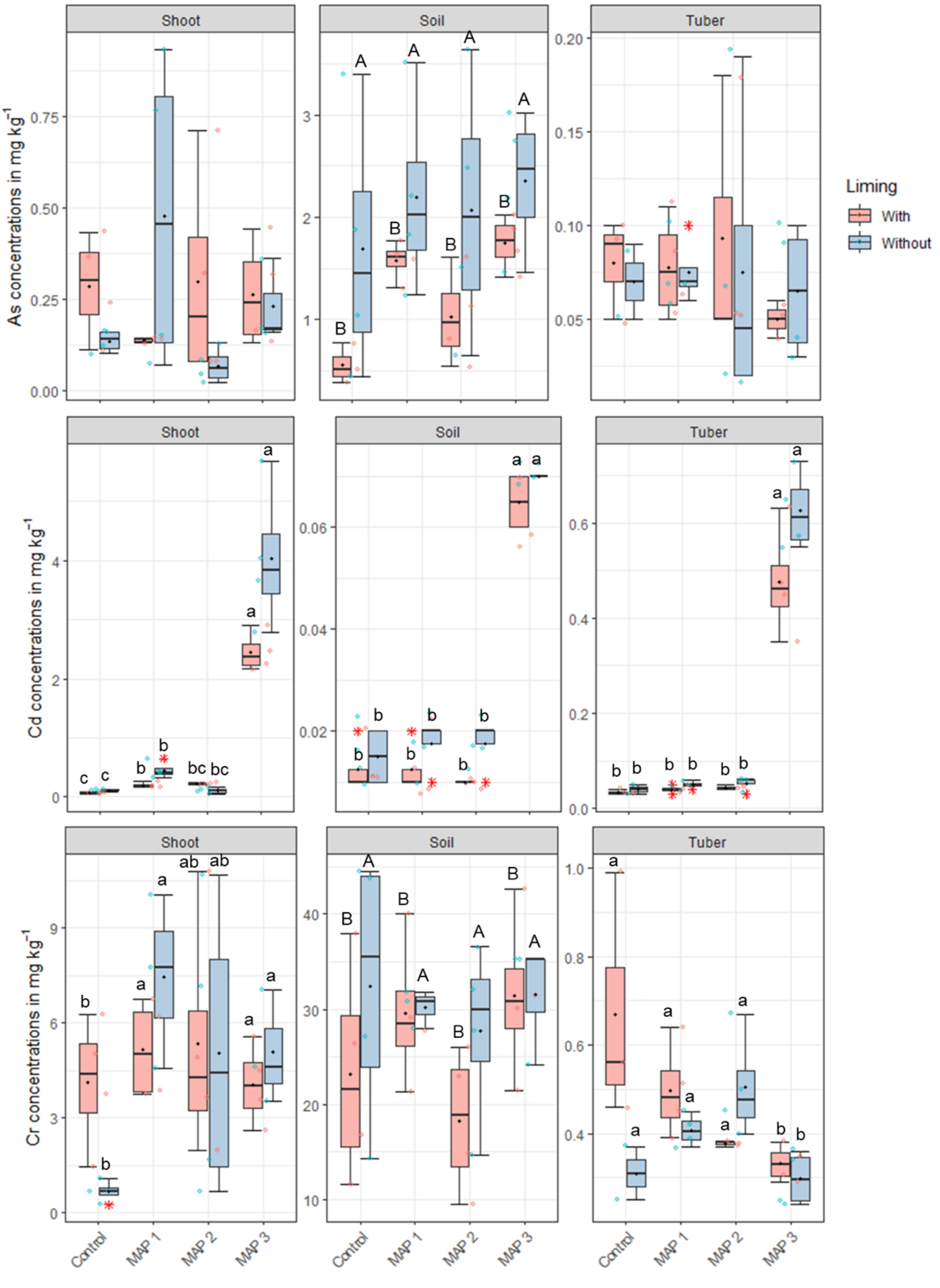


Figure 2. Arsenic (As), cadmium (Cd), and chromium (Cr) concentrations in the soil, shoots, and tubers of potato plants. The data represents a distribution from four replications with a median (black middle line) and means (black points). Uppercase letters represent differences from liming, and lowercase letters represent differences among fertilizer treatments at a probability of 5%, according to Dunn’s test. Missing letters indicate that no difference was found.

Additionally, there is a complex interaction between As and P in the soil-plant system. Unlike the relation with Cd, P addition increases As mobility and availability in soil by competing for adsorption sites releasing As in the soil. However, phosphate can also decrease As uptake and transport by plants due to competition in the rhizosphere (Anawar et al., 2018). Arsenic-P interactions are also affected by As speciation, P requirement by plants, physicochemical properties in the soil, crop species and genotypes, and soil microbial conditions had a huge relevance for these relations (Wu et al., 2022).

Regarding Cd, the mean concentrations in the soil for control and MAP 1 treatments were always above the soil background value (0.4 mg kg^{-1}) (COPAM, 2011). Detection and quantification limits (DL, QL) are summarized in **Table S2**. Soil samples treated with MAP 1 and MAP 2 had concentrations mostly below the DL (0.05 mg kg^{-1}) for the potato experiment (**Fig. 2**). In contrast, soil treated with MAP 3 (high Cd) showed the highest Cd concentrations for all experiments (**Fig. 2, 3, 4**). However, even in MAP 3 treatments, soil Cd concentration after crop growth was much lower than the limit allowed by Brazilian legislation (1.3 mg kg^{-1}), but the concentration in soil was usually lower than the plant tissue concentration, except for the rice experiment. Soil properties like OM, CEC, and pH drive the metal availability in soil. In our case, these characteristics may facilitate Cd uptake by plants, especially crops with high P requirements and Cd uptake.

Similarly to As, a pH effect was observed for Cr in the soil for the potato experiment, where soils without liming (pH 4.82) had 25% more Cr than soils with liming (pH 6.30) (**Fig. 2**). Otherwise, only for the tobacco experiment, we observed significant differences among treatments ($p<0.05$), with higher soil Cr concentrations being found in soil treated with MAP 3, both with (34.18 mg kg^{-1}) and without (32.52 mg kg^{-1}) liming (**Fig. 3**). Significant differences were not observed for Cr soil concentration in the rice experiment (**Fig. 4**).

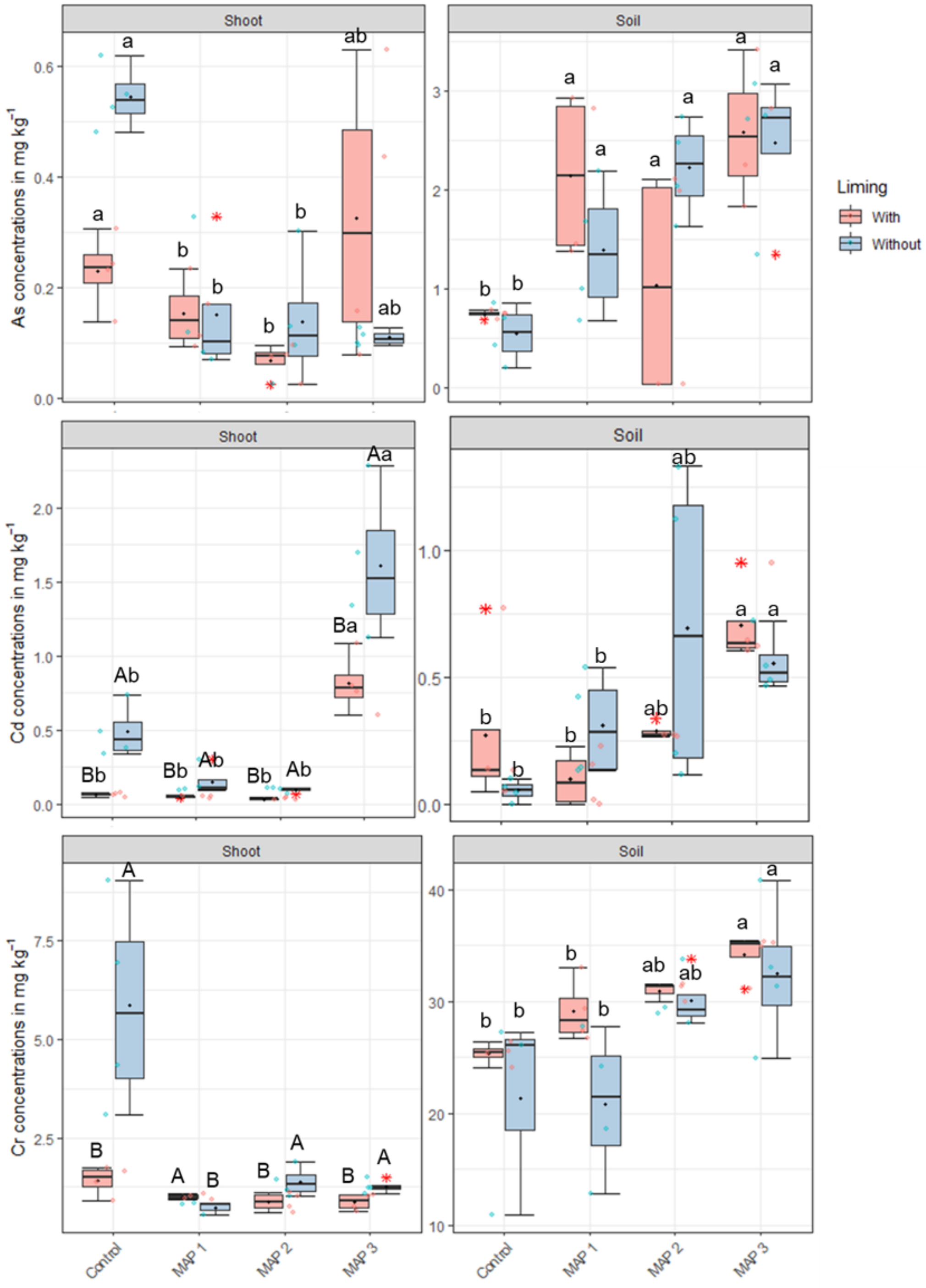


Figure 3. Arsenic (As), cadmium (Cd), and chromium (Cr) concentrations in the soil and shoots of tobacco plants. The data represents a distribution from four replications with a median (black middle line) and means (black points). Uppercase letters represent differences from liming, and lowercase letters represent differences among fertilizer treatments at a probability of 5%, according to Dunn's test.

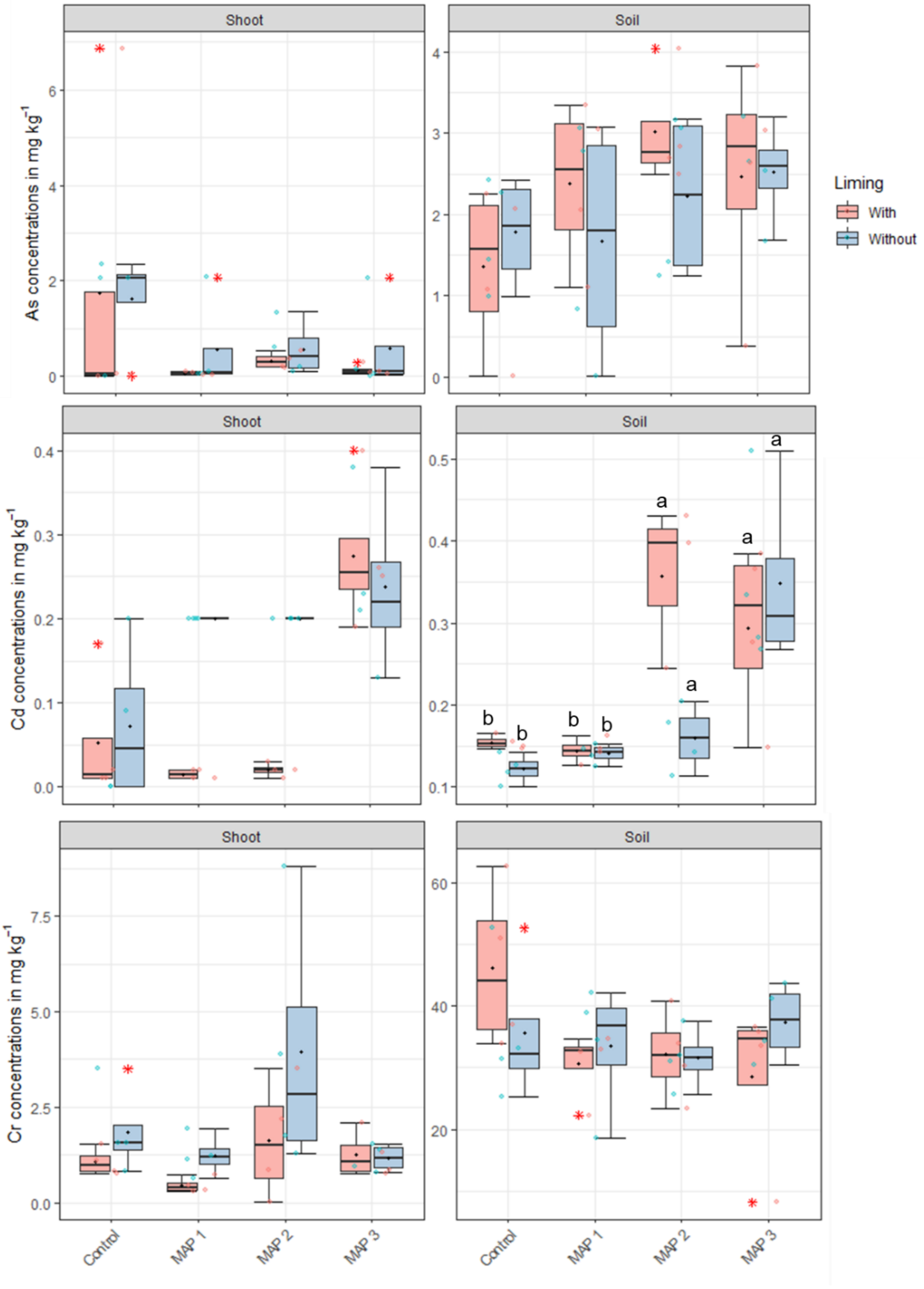


Figure 4. Arsenic (As), cadmium (Cd), and chromium (Cr) concentrations in the soil and shoots of rice plants. The data represents a distribution from four replications with a median (black middle line) and means (black points). Uppercase letters represent differences from liming, and lowercase letters represent differences among fertilizer treatments at a probability of 5%, according to Dunn's test. Missing letters indicate that no difference was found.

Among the PTEs often found in contaminated soils, Cd is known to be highly toxic and readily available for plants due to its high mobility in the soil-plant system (Shahid et al., 2017). In this study, Cd was lower than the prevention value (PV: 1.3 mg kg^{-1}) and investigation value (IV: 3.0 mg kg^{-1}) for agricultural soils established by Normative 420/2009 of the Brazilian legislation (CONAMA, 2009). Although in this study, the Cd content in soil is under the permissible levels in Brazil and elsewhere, in some regions, the Cd content is getting close to the maximum levels allowed for soils (McDowell and Gray, 2022; Suciu et al., 2022), and is increasing over time, which could result in environmental and health risks (Deng et al., 2021).

Similarly, values lower than Brazil's prevention value (PV: 75 mg kg^{-1}) were found for Cr in soil. For As, although a higher pseudo-total concentration was found in the initial characterization of the soil used in this study, the values observed after the treatments were below Brazil's PV for agricultural soils (PV: 15 mg kg^{-1}) as well as the soil guideline value (SGV) for the State of Minas Gerais, Brazil (SGV: 8 mg kg^{-1}) (COPAM, 2011). It is known that the pseudo-total element concentration does not necessarily reflect the element availability because these elements can exist in different oxidation states, which have different solubilities in various environmental conditions. However, unlike As and Cr, which have common forms less (As V and Cr III) and more (As III and Cr VI) soluble and toxic, Cd exists predominantly in soil as an available form for plant uptake (Rahman and Singh, 2019). Additionally, Cr and As have lower plant availability when compared with Cd (Hu et al., 2024).

The results for PTEs in plant tissues varied among elements and crops, with potato plants having the highest P addition. In potato plants, 52.4% more MAP was applied than in tobacco and 64.5% more than in rice plants. So, the PTEs input was higher in potato plants than in tobacco and rice. Besides this, it was noted that Cd was differentially accumulated among plant species. The results of As, Cd, and Cr concentrations are shown in **Fig. 2, 3, and 4**. In tropical soils, Cr occurs predominantly as Cr (III), which is 100-1000 times less toxic than Cr (VI) and more stable and insoluble in tropical soils (Liang et al., 2021; Rahman and Singh, 2019). So, in this study, the Cr in soil was as Cr (III), not resulting in plant toxicity or human health risk. The ranking averages of Cr concentrations in plant shoots for all experiments were 5.57, 1.78, and 1.57 mg kg^{-1} for potato, tobacco, and rice, respectively. No MAP treatment effect was noted for Cr concentration in plant shoots. Regarding plant species, in the MAP 3 treatment, it was applied almost three times more Cr in potatoes than in rice plants, and in rice, shoots were found to have a Cr concentration 7 times smaller than in potato.

MAP 3 was also responsible for loading more Cd into the soil and plant tissues. In the potato experiment, for example, in the MAP 3 treatments, the amount of Cd applied was 0.167 mg Cd kg^{-1} soil, which is 2x and 2.8x more Cd than in the tobacco (0.080 mg Cd kg^{-1} soil) and rice (0.059 mg Cd kg^{-1} soil) experiment, respectively. However, the Cd concentration in potato leaves (4.04 mg kg^{-1} without liming and 2.45 mg kg^{-1} with liming) was 2.5x and 16.8x higher than in tobacco (1.61 and 0.03 mg kg^{-1}) and rice leaves (0.24 and 0.27 mg kg^{-1}), respectively. In contrast, the MAP 1 and 2 values were close to those found for the control (**Fig. 2, 3, 4**). Thus, in the scenario with the highest Cd availability – MAP 3 application and lower pH – rice plants showed the smallest Cd accumulation and potato plants the highest.

Regarding As, the MAP 2 and MAP 3 treatments were responsible for carrying higher As concentrations to the plants, with nearly 2.8x less As input in rice than in potato plants. Conversely, the As concentration in rice was generally smaller than in tobacco and potato, with only plants treated with MAP 2 showing values above the DL (**Table S2**). Soil pH and fertilizer treatments did not affect these concentrations in rice shoots. For the tobacco experiment, arsenic concentrations were lower in plants treated with MAP 1 and MAP 2 compared with the control plants and MAP 3 (**Fig. 2**). It is known that among crops, rice represents a primary dietary source of As in the world due to its high potential to accumulate this metalloid, otherwise, As is lesser transferable from soil to rice grain than Cd (Zhao and Wang, 2020). Our study revealed no grain contamination by these PTEs (data not shown because the values were < the DL for grain samples).

Like the shoots, the potato tubers grown with MAP 3 had a greater Cd concentration (0.63 mg kg^{-1} without liming and 0.47 mg kg^{-1} with liming). At the same time, values below the DL were obtained for MAP 1 **(Fig. 2**). In our study, As and Cr concentrations in potato tubers for all treatments are not different from the control. Corguinha et al. (2012) found that potatoes accumulated more Cd from fertilizers than soybeans due to a higher P requirement of potatoes, which is more than six times what soybeans needed. However, these authors reported that most of the Cd was accumulated in potato peels than in tuber, *i.e.*, almost 20 times more.

Among the PTEs, Cd was the most differentially accumulated in plant tissues, so this element´s concentration was also evaluated in soil xylem and soil solution over time. The results for Cd concentration in sap in this study were correlated with results in shoots, which was similarly observed for rice by Fu et al. (2019). As mentioned for plant shoots, for both crops, potatoes and tobacco, the plants treated with MAP 3 showed the highest Cd concentration in xylem sap in plants without liming (52.05 µg kg^{-1} and 25.31 µg kg^{-1} respectively), with liming reducing this concentration to 39.42 and 19.50 µg kg^{-1}, in that order (**Fig. 5**).

Cadmium is readily loaded into plant tissues, and the xylem and phloem are essential tissues for the distribution of this element in plants, with xylem transport being considered a determinant factor for Cd accumulation in shoots (Fu et al., 2019; Uraguchi et al., 2009). The xylem long-distance transport is also crucial in plant response to Cd toxicity (Luo and Zhang, 2019; Yang et al., 2023). Although the root-to-shoot Cd translocation via xylem and phloem has been investigated recently, to our knowledge, this study is the first to evaluate the xylem loading of Cd from P fertilizers in crops. Our results show that differences in Cd levels in fertilizers are decisive factors for Cd translocation and accumulation in plant tissues.

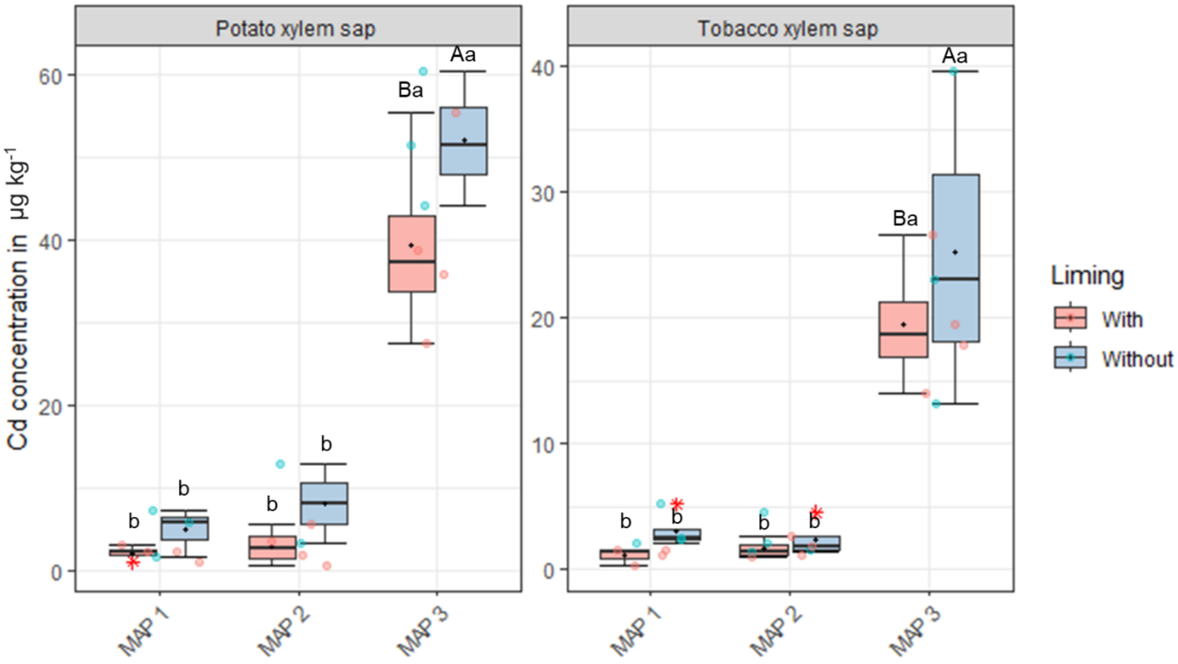


Figure 5. Cadmium concentrations in xylem sap extracted from potato and tobacco plants using the pressure pump method. The data represents a distribution from four replications with a median (black middle line) and means (black points). Uppercase letters represent differences from liming, and lowercase letters represent differences among fertilizer treatments at a probability of 5%, according to Dunn’s test.

Similar results were observed in soil solution (**Fig. 6**). For all the times, MAP 3 (high Cd) was responsible for accumulating more Cd in soil solution showing concentrations above the maximum acceptable level (*i.e.*, 5 µg L^{-1}) proposed by the Brazilian legislation for Cd in groundwater (CONAMA, 2009). MAP 3 treatments with liming had almost ten times more Cd than MAP 1 (low Cd) and >ten times more Cd than that without liming. Mean values for Cd concentration in soil solution for MAP 3 without liming were 12.54, 11.32, 12.26, and 21.10 µg L^{-1} against 1.29, 3.02, 2.16, and 1.95 µg L^{-1} for MAP 1, at 40, 50, 70 and 88 days after planting (DAP) respectively. When liming was applied in soils, these values were reduced to 8.94, 9.02, 10.75, and 8.26 µg L^{-1} for MAP 3, against 0.20, 0.49, 0.67, and 1.05 µg L^{-1} for MAP 1, at 40, 50, 70 and 88 days after planting (DAP) respectively.

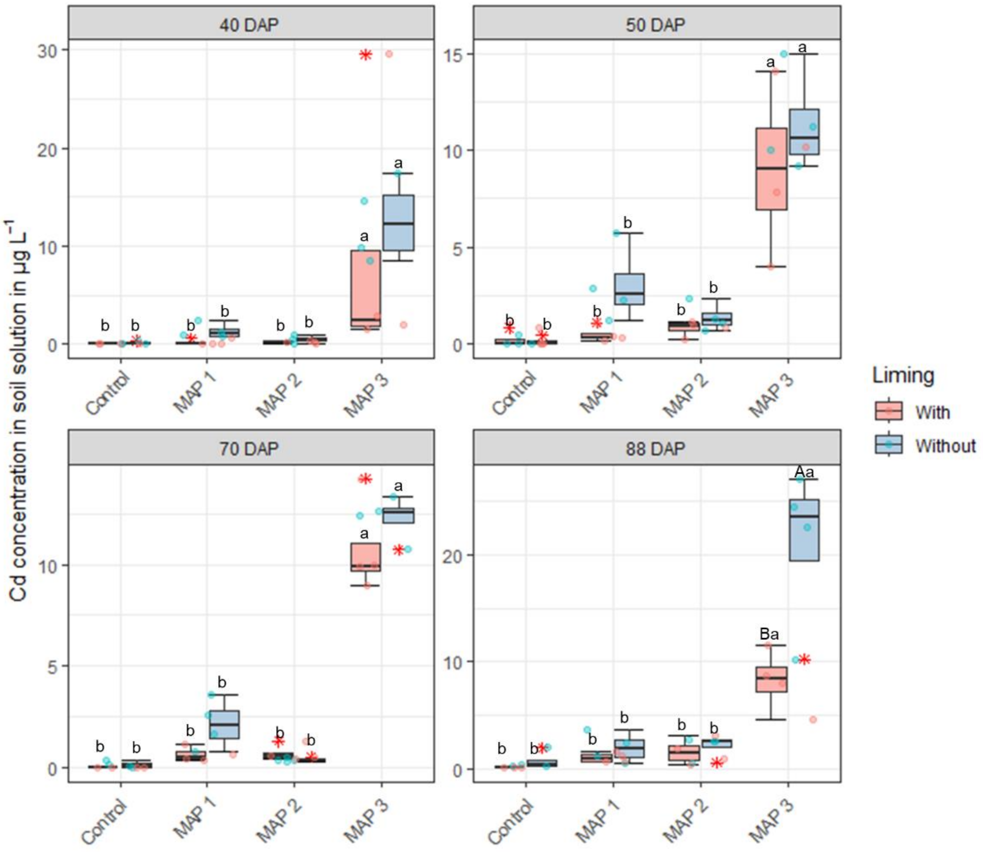


Figure 6. Cadmium concentrations in soil solution extracted from the potato experiment. Data represents a distribution from four replications with a median (black middle line) and means (black points). Uppercase letters represent differences from liming, and lowercase letters represent differences among fertilizer treatments at a probability of 5%, according to Dunn's test. DAP = days after planting.

Although the values obtained in our study are above the Cd concentration usually found in pore water of contaminated agricultural soils (Lavres et al., 2019; Zare et al., 2018), these results are relevant for estimating the long-term impact of high-Cd fertilizer use in agricultural soils. Soil pH was a significant driver of Cd accumulation in soil solution, *i.e.*, the lack of lime increased Cd in the soil extract, resulting in plants` enhanced uptake of this element. Lambert et al. (2007) reported that soil acidification due to fertilizer application enhanced metal solubilization and that Cd concentration in soil extracts treated with an equivalent 15 years of P application increased by >80% compared with the control.

4. Conclusions

Phosphate fertilizers with high Cd content led to increased concentrations of Cd in soil matrix, soil solution, plant tissues, and xylem sap and may result in environmental and food chain contamination if recurred applications are made over time. In this study, there was no accumulation of As or Cr in plant shoots, and the Cr in soil did not represent human health risk. Among the evaluated crops, potatoes showed a higher potential for Cd accumulation. Long-term applications of phosphate fertilizers are potential sources of PTEs in the food chain. Thus, it is essential to establish approaches to pursue soil health and food safety, like using fertilizers with low PTEs levels, especially Cd. Finally, although we have not addressed human or ecological risks in our study, we hope that our findings could help current regulations toward a discussion of green labels or certifications that can help consumers identify safer options in case there is any potential health risk associated with consuming crops grown using P fertilizers containing elevated levels of PTEs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit authorship contribution and statement

Mariana Carvalho: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing Visualization. **Thiago Almeida:** Investigation. **Gustavo Opbergen:** Investigation. **Fábio**

Bispo: Investigation, Formal analysis, Data curation, Writing – Review & Editing, Visualization. **Lívia Botelho:** Methodology, Investigation. **Alexandre Lima:** Methodology, Investigation. **Paulo Marchiori:** Resources, Writing – Review & Editing, Supervision. **Luiz Guilherme:** Conceptualization, Methodology, Validation, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Supplementary table 1: Detection limit (DL), quantification limit (QL), and recovery (%) of total arsenic, cadmium, and chromium from fertilizers, soil, and plant samples submitted to acid digestion assisted by microwave. Recovery % was obtained from mean values of determinations using ICP-MS for As and Cd, and ICP-OES for Cr.

BCR 032 Moroccan Phosphate Rock		**Certified value (mg kg^{-1})**	**Determination value (mg kg^{-1})**	**Recovery %**
Fertilizers MAP	As	9.5	29.81	313.8
	Cd	20.8	16.019 ± 0.692	77
	Cr	257	144.568 ± 6.65	56.2

SRM 2709a San Joaquin Soil		**DL**	**QL**	**Certified value (mg kg^{-1})**	**Determination value (mg kg^{-1})**	**Recovery %**
Potato experiment	As	0.034	0.094	10.5* ± 0.3	7.98 ± 1.090	76
	Cd	0.052	0.138	0.371 ± 0.002	0.29 ± 0.006	79
	Cr	0.237	0.42	130 ± 9	40.2 ± 9.147	31
Tobacco experiment	As	0.05	0.136	10.5* ± 0.3	6.59 ± 0.343	63
	Cd	0.053	1.137	0.371 ± 0.002	0.21 ± 0.015	56
	Cr	1.72	4.571	130 ± 9	54.5 ± 4.473	42
Rice experiment	As	0.203	0.42	10.5* ± 0.3	3.63 ± 3.627	34.5
	Cd	0.05	0.103	0.371 ± 0.002	0.27 ± 0.035	73
	Cr	0.82	1.66	130 ± 9	56.6 ± 5.299	43.5

SRM 1573a Tomato Leaves		**Certified value (mg kg^{-1})**	**Determination value (mg kg^{-1})**	**Recovery %**
Potato shoot	As	0.113 ± 0.002	0.1 ± 0.003	84
	Cd	1.517 ± 0.027	1.13 ± 0.024	75
	Cr	1.988 ± 0.034	0.68 ± 0.617	34
Potato tuber	As	0.113 ± 0.002	0.12 ± 0.012	107
	Cd	1.517 ± 0.027	1.43 ± 0.067	94
	Cr	1.988 ± 0.034	1.32 ± 0.055	66
Tobacco shoot	As	0.113 ± 0.002	0.094 ± 0.018	84
	Cd	1.517 ± 0.027	0.917 ± 0.087	60.5
	Cr	1.988 ± 0.034	1.28 ± 0.172	64.2
Rice shoot	As	0.113 ± 0.002	0.055 ± 0.040	49
	Cd	1.517 ± 0.027	0.81 ± 0.104	53
	Cr	1.988 ± 0.034	1.35 ± 0.111	68

DL and QL (detection and quantification limits in mg kg-1), Certified values ± standard deviation, Determined values = mean values, Recovery (%) = (determined/certified values) x 100. * Reference values for elements in SRM 2709a.

Supplementary table 2: Soil pH evaluation over time on treatments with and without lime application. Values are means followed by the standard error.

pH treatment	**8 DAL**	**16 DAL**	**45 DAL**	**58 DAL**
Without liming	5.164 ± 0.123	5.120 ± 0.053	4.643 ± 0.082	4.821 ± 0.041
With liming	6.445 ± 0.084	6.273 ± 0.100	6.137 ± 0.067	6.302 ± 0.030

DAL = days after liming

Manuscript 2: Differences in growth, biochemical, and ionomic responses to P fertilizers with various metal(loid)s content and liming among crops

Manuscript under submission in Frontiers in Plant Science (Impact factor: 5.6)

Abstract

Avoiding the occurrence of undesirable levels of cadmium (Cd) and other toxic metal(loid)s in phosphate fertilizers is a matter of great concern in many agroecosystems, especially in tropical regions. This study investigated physiological alterations in crop response to different levels of pH and the toxic metal(loid)s arsenic (As), cadmium (Cd), and chromium (Cr) present in phosphate fertilizers of different origins. Three plant species (potato, tobacco, and rice) were cultivated in pots containing soil with and without liming using three different monoammonium phosphate fertilizers (MAP), as a phosphorus (P) source for plants growing on tropical soil. Growth, yield, fluorescence of chlorophyll, antioxidant system, nitrogen compounds, and soluble sugar content were evaluated. For each experiment, we analyzed bioaccumulation of As, Cd, Cr, and different macro and micronutrients - boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S), and zinc (Zn) - in soil and plant shoots. Our findings indicated that plant physiology was mostly affected by low pH rather than the metal(loid)s available in the fertilizers. Acidic soils decreased plant growth, yield, and sugar content, and induced metabolic adjustments related to antioxidant enzymes and proline. The levels of all elements evaluated were affected by liming differentially among the plant species. The higher level of Cd in MAP 3 reduced Mn and Zn levels in plant shoots for potatoes and tobacco while inducing an increase of antioxidant enzymes and sugars in rice. This study indicated that the metal(loid)s content in fertilizers was not enough to induce damage to the growth and development of plants, yet metabolic adjustments were observed in rice. Rice also had a lower Cd translocation compared with potato and tobacco, followed by a higher tolerance to acidic pH.

Keywords: acidity, liming, nutritional imbalance, phosphate fertilizers, toxic metal(loid)s.

1. Introduction

Soils contains many mineral elements, some of these are essential for plants and animals, and others are nonessential and even potentially toxic for both. Arsenic (As), cadmium (Cd), and chromium (Cr) are three of the five most common toxic metal(loid)s (hereafter called trace metals - TMs, for simplicity) that pose a risk to human health (Mood et al., 2021). However, phosphate (P) fertilizers often play a major role in the bioavailability of these TMs for agricultural soils (Gupta et al., 2014; Hu et al., 2024).

Although plant cell membranes are specialized in constraining the uptake and translocation of harmful substances, TMs can be taken up by roots through plant nutrient transport systems using different mechanisms. The As uptake depends on the speciation in soil and usually is highly retained in roots (Zhao and Wang, 2020), but moves through the P transporters (Finnegan and Chen, 2012). Like As, Cr uptake is also affected by soil speciation, with phosphate and sulfate transporters being used to load Cr (VI) for cells (Singh et al., 2013). On the other hand, Cd is easily taken and translocated via divalent cation transporters (Clemens and Ma, 2016), and is often found in xylem sap in high levels (Riaz et al., 2021).

Besides TMs concentration and availability in agriculture soils, soil pH is a major factor driving TMs uptake by plants, especially Cd (Gupta et al., 2019). Acidic pH is strongly and positively correlated with Cd solubility and mobility in soils (Sheoran et al., 2016). In addition to increasing Cd loading to plants, a low pH also poses constrain for plant growth, productivity, physiological parameters, and nutritional status (Jovovic et al., 2021; Long et al., 2017; Wang et al., 2022).

Hence, liming is an effective strategy to cope better with high Cd in soil (Zhao and Wang, 2020) and improve soil and plant health (Bian et al., 2013). Phosphate also can be used to immobilize Cd in soils efficiently (Liao et al., 2023). However, Cd and other TMs are commonly found as trace elements in phosphate fertilizers (Verbeeck et al., 2020). Thus, the use of low Cd P-fertilizers combined with an increase in soil pH is essential to reduce Cd accumulation in areas in which this metal represents a safety risk.

In plant leaves, these TMs may result in growth retardation, ROS production, antioxidant enzymatic activation, and damage in CO_2 assimilation, in the activity of photosystem II and the electron transport chain (Jin et al., 2024; Nabi et al., 2021; Souri et al., 2019; Finnegan and Chen, 2012; Sharma et al., 2020). The increase in the content of soluble sugars due to a misbalance in related enzymes is reported in these TMs in several crops (Li et al., 2020; Shahid et al., 2019; Xie et al., 2014).

Plants also may accumulate soluble sugars as a strategy to deal with toxic concentrations of TMs (Li et al., 2020), as well as other osmoregulatory substances like proline (Zdunek-Zastocka et al., 2021). Furthermore, due to chemical analogies, these TMs can replace macro and micronutrients affecting all plant primary metabolism (Feng et al., 2017; Khan et al., 2016, 2019; Naeem et al., 2019; Teles et al., 2022).

Among crops, vegetables, and cereals are major sources of TMs in the food chain (Corguinha et al., 2015). Potato and rice are staple foods that represent relevant sources of intake of TMs, specifically Cd (Corguinha et al., 2015; Duan et al., 2017; Mengist et al., 2017; Zhao and Wang, 2020; Ye et al., 2020). Besides, tobacco is also an important economic cash crop and is a potential source of Cd intake for humans (Mei et al., 2022).

Therefore, this work focused on investigating how plants of potatoes, tobacco, and rice respond to different concentrations of arsenic, cadmium, and chromium from P fertilizers (MAP), and how liming application impacts these physiological responses. Our hypothesis was that higher arsenic, cadmium and chromium concentrations in P fertilizers would affect the P and other nutrients uptake by plants, altering the plant ionomic and limiting carbohydrates metabolism, photosynthesis, and energy, which reduce the plant growth and yield.

2. Materials and Methods

2.1. Plant materials and growth conditions

The experiments were conducted in 2021 and 2022 at the Department of Soil Science, Federal University of Lavras, Lavras, Minas Gerais, Brazil. The CMG 1590 rice cultivar, the CSC4704 tobacco cultivar, and the Asterix potato cultivar were used to investigate physiological and nutritional disturbances due to soil pH and metal bioaccumulation in plants from P fertilizers (monoammonium phosphate - MAP) applied in a Red-Yellow Latosol with a clay fraction of 32%.

The MAP fertilizers used were from three different geographic regions and had different toxic metal content as described in **Table S1,** where the MAP 3 show Cd concentration 14-fold MAP 2 and 20-fold MAP 1. For each experiment, the experimental design was laid out in a randomized block design with three MAP fertilizers and two pH levels (with liming and without liming).

The soil was homogenized and submitted to two treatments: native pH (pH 5.1), and soil with liming (pH 6.3), which was performed following a 60-day incubation period. The soil pH was monitored in water with a pH Meter using a soil: solution ratio of 1:2.5 after shaking and 1-hour rest before liming, during incubation time, and after crop harvesting. After incubation, fertilization for three crop experiments was performed according to Malavolta (1980), De Oliveira et al. (2019), Boldrin et al. (2013), as described in Carvalho et al. (2024) (submitted). Three experiments were conducted in a greenhouse during all the life cycles of crops, *i.e.*, 77 days for potato, 165 days for tobacco, and 170 days for rice.

2.2 Bioconcentration and translocation of metal(loid)s for potato, tobacco, and rice

Bioconcentration factor (BCF) refers to metal accumulated in plant tissue that was determined in the soil as shown below (Boim et al., 2016; Singh et al., 2010):

Shoot bioconcentration factor: $BCF_{shoot} = C_{shoot} / C_{soil}$ (1)

Where C_{shoot} is the element concentration in shoot, and C_{soil} is the element concentration in soil.

The translocation factor (TF) was calculated for potatoes tubers as:

Tuber translocation factor: $TF_{tuber} = BCF_{tuber} / BCF_{shoot}$ (2)

Where BCF_{tuber} is the result of C_{tuber} / C_{shoot} and BCF_{shoot} represents the result of C_{shoot} / C_{soil}.

2.3 Measurements of phytotechnics parameters of potato, tobacco, and rice plants

The growth and development were monitored over the plant life cycle for potato, tobacco, and rice, and the measurements at the end of the experiment were chosen to represent plant responses to treatments.

For the potato experiment, biometrics variables such as stem height, stem diameter, shoot dry biomass, number of leaves, and folioles were measured, also as productivity was evaluated by tuber number and dry biomass. Tobacco growth was also evaluated by biometrics variables: stem height, stem diameter, and number of leaves. And, finally, the growth and development of rice plants were measured by plant height, tiller, panicle numbers, shoot, and root dry biomass, as well as productivity, which was evaluated by the number and dry biomass of grains.

2.4 Chlorophyll a fluorescence

All measurements were taken on fully expanded leaves for potato, tobacco, and rice plants. For the potato experiment, the parameters were obtained using MultispeQ fluorometer (Kuhlgert et al., 2016) during the morning and night to investigate fluorescence yield changes in light-exposed (LE) and dark-adapted (DA) leaves, respectively. The measuring protocol (Photosynthesis RIDES) illuminated the leaves with photosynthetic photon flux density

(PPFD) matching that of the incident light to generate the initial fluorescence of LE and DA leaves - F0’ and F0; stade-state fluorescence – Fs; and the maximum fluorescence of LE and DA leaves - Fm’ and Fm. These same parameters were obtained from tobacco and rice plants with a PAM fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) using a leaf clip holder and 30 minutes of dark adaption.

Calculations of photochemical quenching were carried out as follows: maximum quantum yield of PSII in LE leaves – *Fv’/Fm’* = *(Fm’-F0’)/Fm’* and DE leaves – *Fv/Fm* = *(Fm-F0)/Fm*; effective quantum yield of PSII – ϕPSII = *(Fm’-Fs)/Fm’* according to Genty et al. (1989). The ϕPSII is used to calculate electron transport rate – ETR = (ϕPSII*PPFDa*(0.5), where PPFDa is assumed absorbed light (μmol photon m^{-2} s^{-1}) (Genty et al., 1989). The proportion of open PSII – qP = (Fm’-Fs)/(Fm’- F0’) was calculated as described by Maxwell et al. (1994). Non-photochemical quenching was obtained from NPQ = (Fm-Fm’)/Fm’ (Bilger and Björkman, 1990), and qL = qP*(F0’/Fs) referred to photoinhibition (Osmond, 1994).

2.5 Biochemical analysis – quantification of hydrogen peroxide and antioxidant enzymes

Leaf hydrogen peroxide (H_2O_2) content was determined by Velikova et al. (2000). Briefly, 0.2 g of fresh leaf was grounded with liquid nitrogen and mixed with 1.5 mL of trichloroacetic acid 0.1%. The samples were centrifuged at 12,000 g for 15 minutes and the reaction started with the addition of 45 µL of 10mM potassium phosphate buffer (pH 7.0), and 90 µL of 1M potassium iodide. The readings were carried out in a spectrophotometer at 390 nm and the H_2O_2 content was determined using a standard curve.

To obtain the crude protein extract for the quantification of total proteins content, and activity of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), 0.2 g of leaf tissue was macerated in liquid nitrogen with polyvinylpolypyrrolidone (PVPP). The extraction was performed according to Biemelt et al. (1998) with 1.5 mL of extraction buffer (potassium phosphate 400 mM pH 7.8 + EDTA 10 mM + ascorbic acid 200 mM), followed by centrifugation at 13,000 g during 10 minutes at 4 °C. SOD activity was quantified according to Giannopolitis and Ries (1977), CAT activity was quantified according to Havir and McHale (1987), and APX activity according to Nakano and Asada (1981).

2.6 Quantification of proline

The method described by Torello and Rice (1986) was used for proline quantification, where 0.2 g of dry material was homogenized with 10 mL of 3% sulfosalicylic acid and centrifuged at 5,000 rpm for 20 minutes. After, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were mixed with 2 mL of supernatant, followed by 1 hour in a water bath at 100 °C. The proline content was determined using a standard curve (Bates et al., 1973).

2.7 Quantification of macromolecules — total soluble sugars, reducing sugars, starch, sucrose, amino acids, and protein content

The methodology adapted by Zanandrea et al. (2010) was used to extract macromolecules. To do this, 0.2 g of dry leaf tissue was homogenized with 10 mL of 0.1M potassium phosphate buffer pH 7.0, followed by 30 minutes in a water bath at 40 ºC. The samples were centrifuged at 5,000 rpm for 10 minutes, the pellet from the extraction was used to quantify starch, and the supernatant was collected for the following analysis. Total soluble sugars (TSS), sucrose, and starch were quantified by the anthrone method according to Dische (1962) and reducing sugars (RS) by the DNS method according to Miller (1959). Amino acids were quantified by the ninhydrin method according to Yemm and Coccking (1954), and protein content by the method described by Bradford (1976).

2.8 Ionomic analysis

An Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was used to determine the total concentrations of essential (B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) and nonessential (Cr) elements in soil and shoot tissue. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine the total concentration of nonessential elements As and Cd in soil and shoot tissue for the three crops: potato, tobacco, and rice.

The soil and plant dry samples (0.5 g) were added to Teflon tubes and digested in a microwave oven (MARS-5 ®, CEM) with 5 mL of concentrated HNO_3, using USEPA Method 3051A (Environmental Protection Agency, 1998). After digestion, the solutions were cooled, filtered through Whatman no. 40 filter paper, and received a volume of 5 mL of distilled water. The accuracy was checked by using blanks and standard reference material for soil (SRM 2709 San Joaquin soil; Light Sandy Soil BCR No. 142), and for plant (White Clover (BCR 402, IRMM, Geel, Belgium; Spinach Leaves NIST 1570, Tomato Leaves NIST 1573a, and Peach Leaves NIST 1547) throughout digestion and analysis.

2.9 Statistical analysis

All samples were subjected to statistical evaluation on the software R 4.2.3 ($p < 0.05$) (R Development Core Team, 2018). The Shapiro-Wilk test was used to test the normality and the homogeneity of variance for data, and log transformations were conducted when necessary for the use of ANOVA followed by Duncan's test. Since the assumptions were not met even after data conversion, the non-parametric Wilcox and Kruskal-Wallis tests for multiple group comparison were used, followed by Dunn's test. Correlations plots for concentrations of elements in shoots of potato, tobacco, and rice for all three MAP fertilizers, with and without liming were performed on the software R 4.2.3 ($p < 0.05$) (R Development Core Team, 2018) applying the Pearson test with regression significance for $P < 0.05$.

3. Results

3.1 Bioconcentration and translocation factor of metal(loid)s for potato, tobacco, and rice

Plants have different strategies to deal with nonessential elements, such as toxic metal(loid)s (TMs). The details of TMs concentrations in MAP fertilizers are shown in **Table S1**. Concentrations in MAP fertilizers are in the following order MAP 3 > MAP 2 > MAP 1, with Cd showing the most contrasting concentrations among fertilizers. Similarly, the accumulation of this metal was higher in plants grown with this MAP (Figure 1). For potato and tobacco shoots the bioconcentration factor (BCF) was > 30 in MAP 3 treatment, while a 2-fold and 3-fold less content was observed for MAP 1 treatment in both, potato (Figure 1a), and tobacco (Figure 1c), respectively. A liming effect was observed for Cd as well as Cr concentration in tobacco plants. For rice plants, the BCF was < 1.5 in all treatments, with no difference between MAP fertilizers in most acidic soils, yet MAP 3 presented a higher value in limed soils, relative to the other fertilizers (Figure 1d).

3.2 Phytotechnics parameters of potato, tobacco, and rice plants

The plant growth and yield was evaluated in response to treatments of liming and MAP fertilizers. A most acidic soil pH reduced the number of leaves (Figure 2a, b) and the number of tubers as well as its biomass (Figure 2e, f). Similarly to potatoes, tobacco plants had stem height, diameter, and leaf number reduced under these conditions (Figure 2g, h, i). However, in rice plants, only tiller numbers showed a small reduction in response to soil pH (Figure 3h). No MAP treatment effect was observed for none of these parameters.

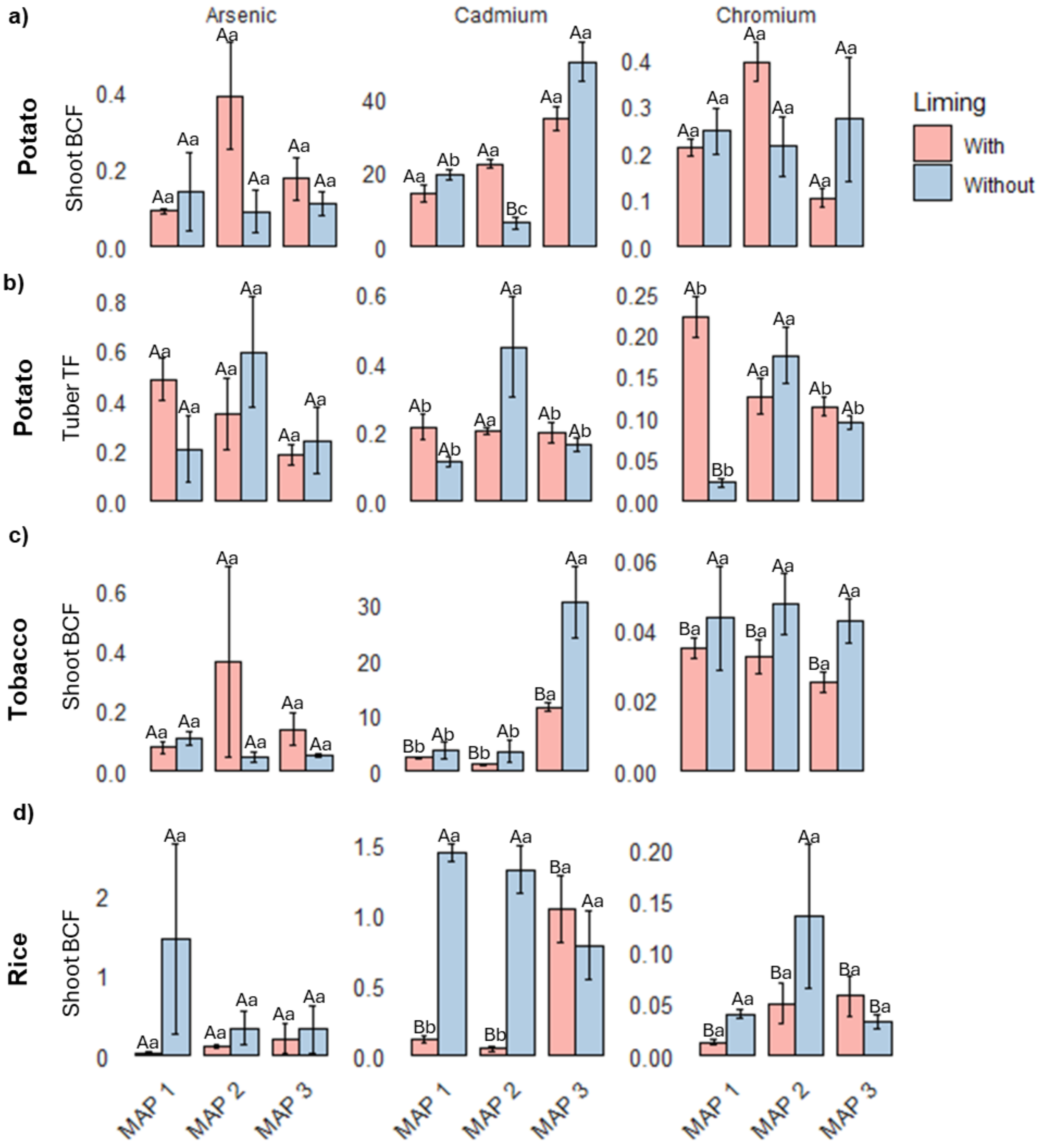

Figure 1: Shoot bioconcentration factor (BCF) for As, Cd, and Cr in potato (a), tobacco (c), and rice (d) plants; and tuber translocation factor (TF) for As, Cr, and Cr (b) in response to MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

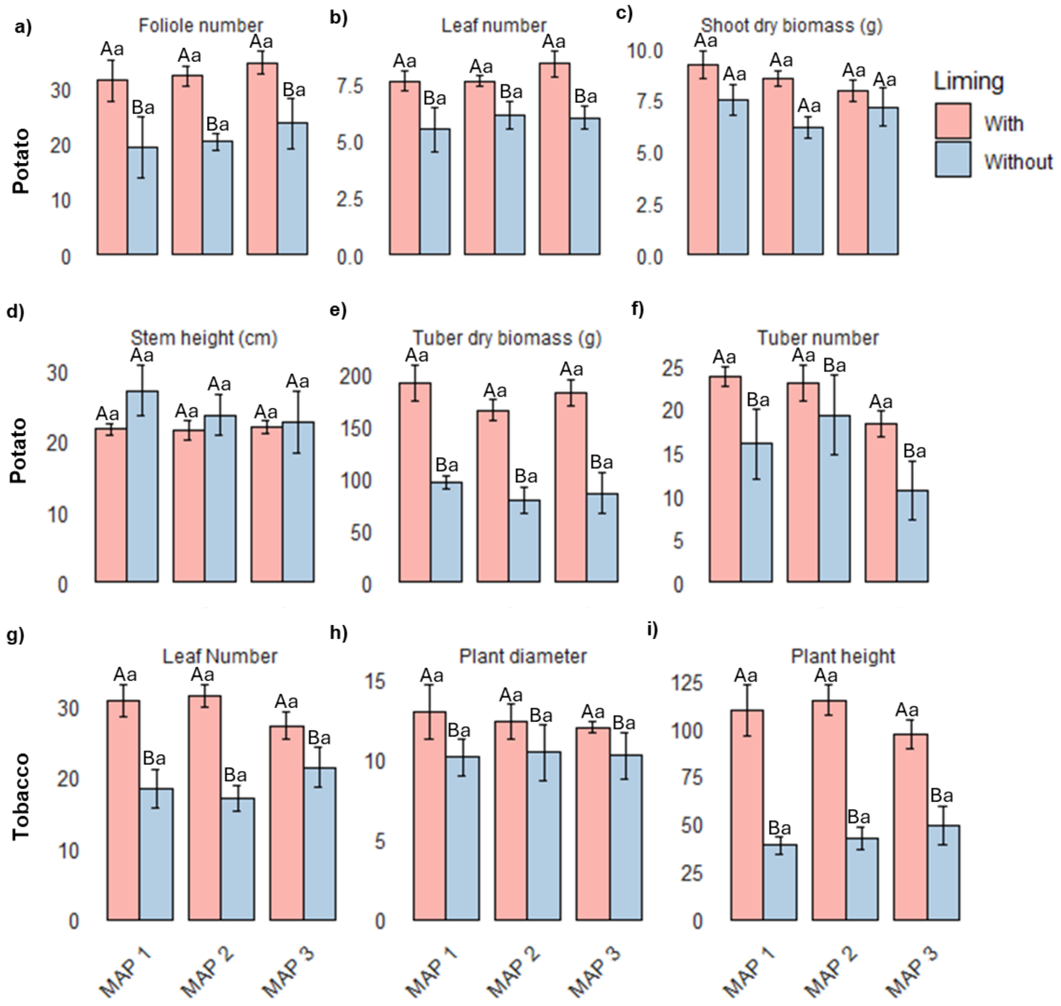

Figure 2: Number of folioles (a), leaves (b), shoot dry biomass (c), stem height (d), tuber dry biomass (e), and tuber number (f) for potato plants in response to liming and MAP treatments. Leaf number (g), plant diameter (h), and height (i) for tobacco plants in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan’s test ($p < 0.05$).

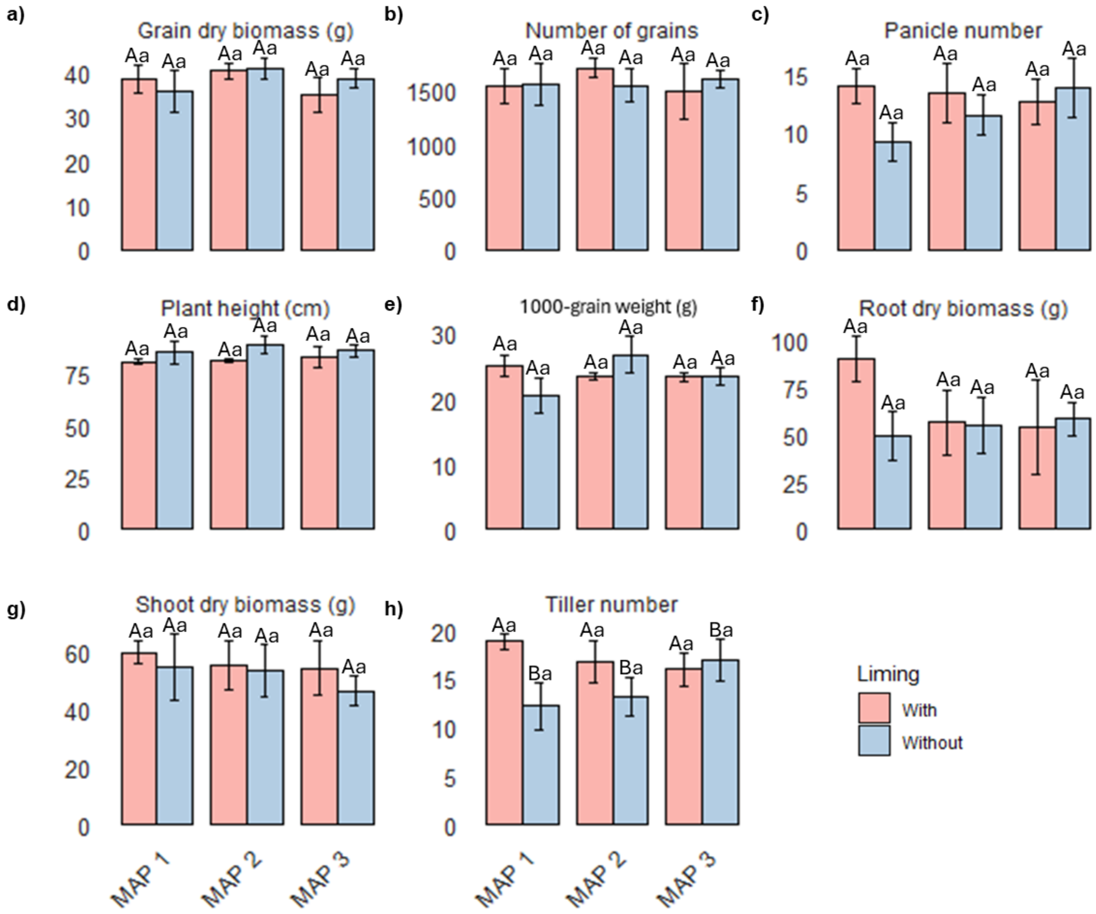

Figure 3: Biomass and number of grains (a, b), number of panicle (c), height (d), 1000-grain weight (e), dry biomass of root (f) and shoot (g), and tiller number (h) of rice plants in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

3.3 Chlorophyll a fluorescence

The electron transport rate (ETR), and maximum quantum yield of PSII in dark-adapted leaves (Fv/Fm) did not show significant differences among the treatments in each experiment (Figure 4a, c, j, h, n, p). The maximum quantum yield of PSII in light-exposed leaves (Fv'/Fm') showed an interaction between liming and MAP treatments just for potato plants. Plants grown with MAP 1 showed a reduction in soil without liming (Figure 4b). No alterations were observed for other plants (Figure 4i, o).

The effective quantum yield (ϕPSII) showed an interaction between liming and MAP treatments for all experiments. Potato plants had ϕPSII decreased in plants growing in soil without liming for all MAP treatments, and for this treatment, MAP 1 presented the lower value (Figure 4d), which was followed by a higher non-photochemical quenching (NPQ)

(Figure 4e). A liming effect was also observed for photochemical quenching related to photoinhibition (qL) in potato plants (Figure 4f), and the photochemical coefficient related to open PSII (qP) did not show alterations (Figure 4g). Tobacco plants presented a liming effect for ϕPSII and qP only in treatments with MAP 2 and MAP 3 (Figure 4k, m), with MAP 1 showing a higher qP value in plants without liming (Figure 4m). No alterations were observed in qL (Figure 4l). In rice plants, no liming effect was observed for ϕPSII (Figure 4q), qL (Figure 4r), and qP (Figure 4s). However, ϕPSII and qP were lower in plants grown with MAP 1.

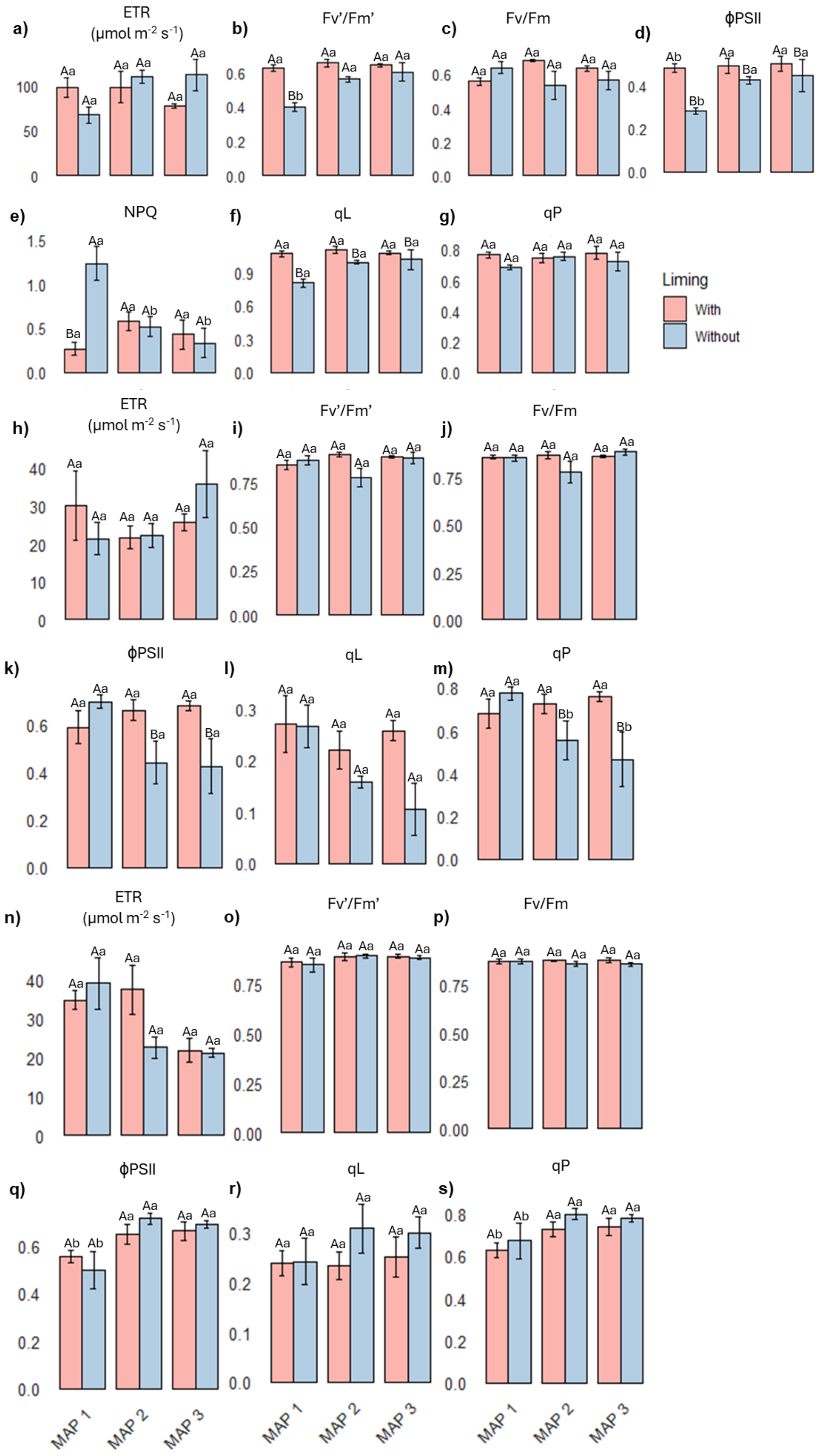

Figure 4: Electron transport rate – ETR (a, h, n); maximum quantum yield of PSII in light-exposed (b, i, o) and dark-adapted (c, j, p) leaves; effective quantum yield of PSII (d, k, q); non-photochemical quenching (e); photochemical quenching related to photoinhibition (f, l, r); photochemical coefficient related to open PSII (g, m, s) potato, tobacco, and rice plants, respectively, in response to liming and MAP treatments. Values are means of four replications

± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

3.4 Biochemical analysis – quantification of hydrogen peroxide and antioxidant enzymes

Our results showed different biochemical responses to soil pH and MAP treatments among the species. Both liming and MAP interacted to affect hydrogen peroxide (H_2O_2) concentration in potato plants (Figure 5a). The H_2O_2 content in the leaves of potato plants decreased without liming, and in these conditions, the level was as follows MAP 2 > MAP 3 > MAP 1, whereas the values for liming condition were similar among MAP treatments. The SOD and CAT activity in the potato leaves was higher in plants growing without liming, compared with liming application for all MAP treatments (Figure 5b, c). No significant differences were observed for APX activity in response to liming or MAP (Figure 5d).

The H_2O_2 concentrations in tobacco leaves did not differ significantly among treatments, but they were almost 20 times higher than the concentration in potato and rice leaves (Figure 5e). No difference was observed for enzymatic activities (Figure 5f, g, h). The only exception was the lower SOD activity in MAP 1 with liming (Figure 5f). For rice plants, in plants grown with liming a higher level of H_2O_2 was observed in MAP 1, followed by MAP 2 and 3, whereas without liming MAP 3 showed a higher concentration, with liming interaction (Figure 5i). Moreover, this treatment showed greater SOD activity (Figure 5j), and greater activity of SOD, CAT, and APX was observed without liming (Figure 5j – l).

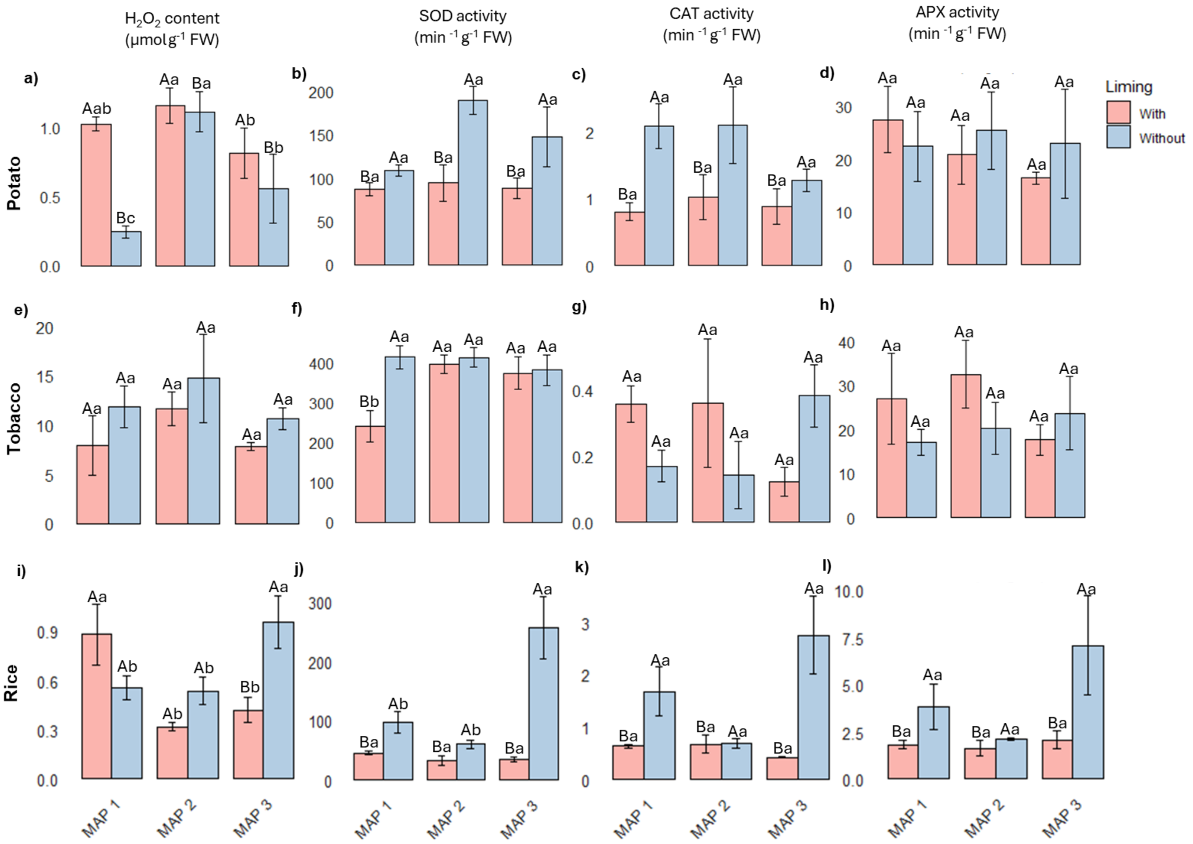

Figure 5: Hydrogen peroxide content (H_2O_2) in potato (a), tobacco (e), and rice (i) plants in response to liming and MAP treatments. The activity of superoxide dismutase (b, f, j), catalase (c, g, k), and ascorbate peroxidase (d, h, l) in plants of potato, tobacco, and rice, respectively in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

3.5 Quantification of proline

The proline content was significantly altered as a result of soil pH only in tobacco plants with plants grown without liming showing greater production (Figure 6b). Proline content in rice plants did not differ among treatments (Figure 6e).

3.6 Quantification of macromolecules — amino acids, protein, reducing sugars, total soluble sugars, starch, and sucrose content

The treatments without liming increased the amino acids while decreasing protein content in tobacco leaves (Figure 6a, c). Amino acids did not differ among treatments, while protein content decreased without liming for rice plants (Figure 6d, f). The plants also presented different responses in carbohydrate concentrations in response to liming and MAP treatments, but in general higher concentrations were observed in plants grown with liming.

Regarding potato leaves, soluble sugars total (SST) and sucrose content decreased in treatments without liming (Figure 7b, d). For starch content, only MAP affected the results, with the highest value observed for MAP 2 (Figure 7c). No significant differences were observed in reducing sugar content (RS) (Figure 7a). As shown in Figure 7e – h, the treatment without liming decreased RS, SST, starch, and sucrose levels in tobacco leaves. In rice plants was observed interaction between liming and MAP treatments. Reducing and total sugars were not impacted by lime, and the highest concentration was observed in MAP 3 (Figure 7i, j). MAP 3 was also responsible for a higher concentration of sucrose (Figure 7l). Moreover, higher concentrations of sucrose and starch were observed with liming (figure 7k, l).

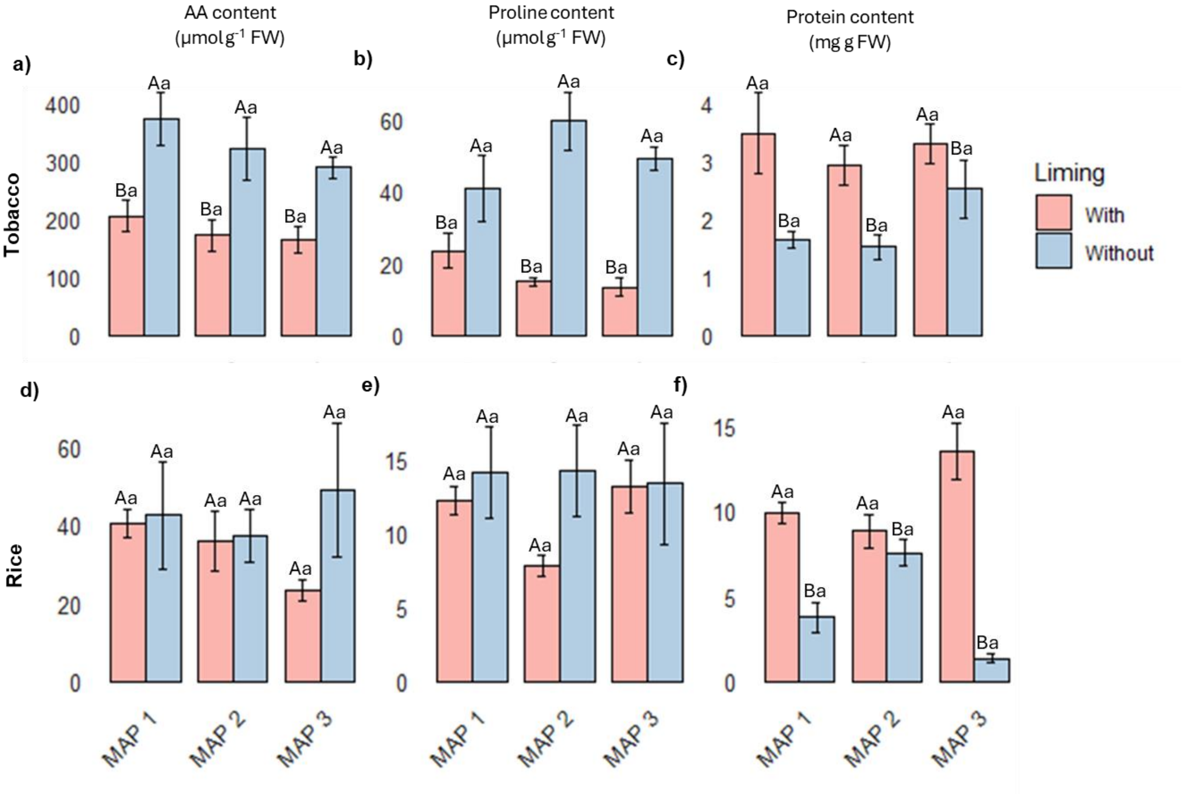

Figure 6: Amino acids (a, d), proline (b, e), and protein content (c, f) in tobacco and rice plants, respectively, in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

3.7 Ionomic analysis

The concentration of essential elements in soil and plant shoots for potato, tobacco, and rice experiments showed different patterns. Regarding the potato experiment, soil without liming revealed higher concentrations of K, Mn, and P after plant growth, and MAP 3 treatment showed the highest Mn level compared with other MAPs (Figure 8). The liming effect also was observed for the tobacco experiment in soil concentrations of Ca, and S just in MAP 3 treatment (Figure 8). Moreover, MAP 3 was responsible for the highest soil concentrations of Fe, S, Mn, P, and Zn compared with other treatments. In rice growth, the soil without liming had higher concentrations of S and K. No MAP effect was observed for mineral elements in the soil.

Nutrient concentrations in plant shoots are shown in Figure 9, for potato, tobacco, and rice, respectively. For potato plants, the shoot level of Ca and Mg decreased upon decreasing soil pH in soils without liming, whereas Cu, S, K, P, Mn, and Zn levels increased in these conditions. Only Fe levels in potato shoots were affected by MAP treatments, and MAP 2 had the lowest value. Regarding the tobacco experiment, B, S, and Mn levels in the shoot were decreased without liming, while Ca, Cu, K, and Zn levels increased with pH decreasing. MAP treatments influenced only Mn levels in tobacco shoots, with MAP 3 showing the lowest value. In the rice experiment, just the S level decreased with a pH reduction in soils without liming, whereas Cu, Fe, Mn, and Zn levels increased in these conditions. Among the MAP treatments, the shoot concentration of Fe was higher in MAP 2, followed by MAP 3 and MAP 1.

3.8 Correlations among the PTEs and nutrients in plant shoot

Figures 10, 11, and 12 showed the correlations among elements in two soil pH for potato, tobacco, and rice, respectively. It could be observed that without liming growth conditions and potato experiment had more correlations than others. In general, As, Cd, and Cr were positively correlated, as well positively correlations with Cu, Fe, and P were often found. Correlations between Cd and Zn were observed for tobacco and rice experiments, only with liming (Figures 11f, 12d), and between Cd and Mn varied between positive correlation in tobacco and negative in rice experiments, both without liming (Figures 11a-12e).

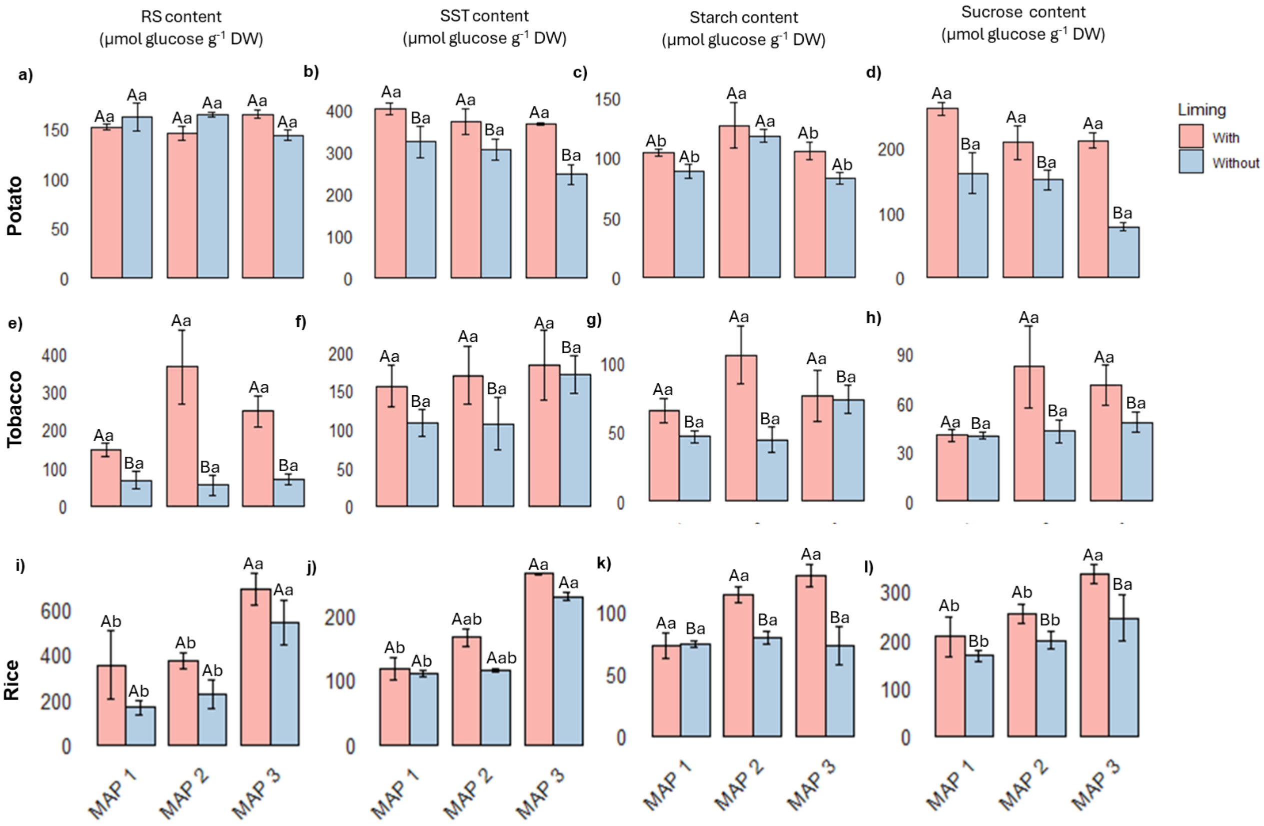

Figure 7: Reducing sugars (a, e, i), soluble sugars total (b, f, j), starch (c, g, k), and sucrose content (d, h, l) in potato, tobacco, and rice plants, respectively, in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

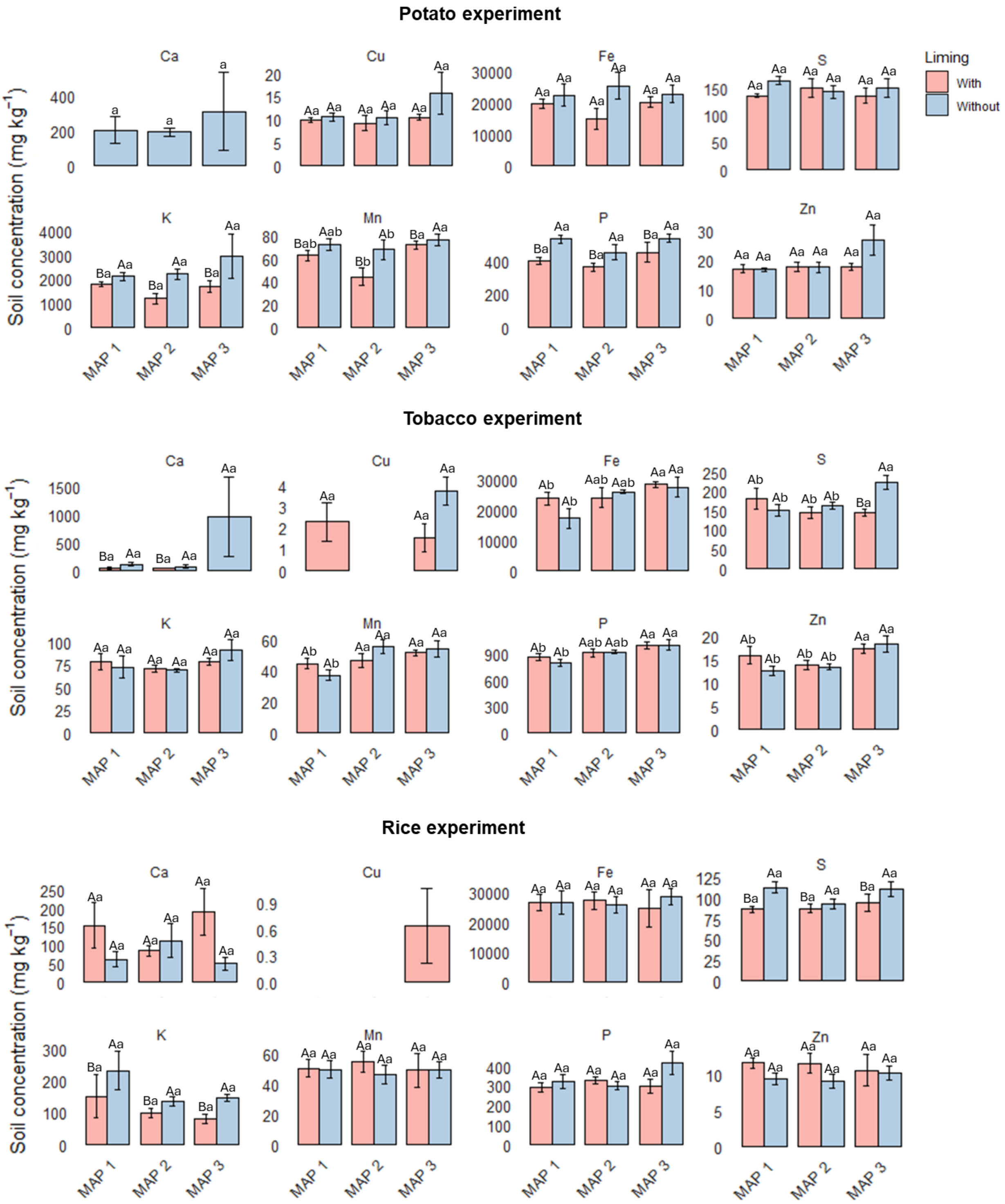

Figure 8: Macro and micronutrient concentrations in soil for potato, tobacco, and rice growth, respectively, in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan’s test ($p < 0.05$).

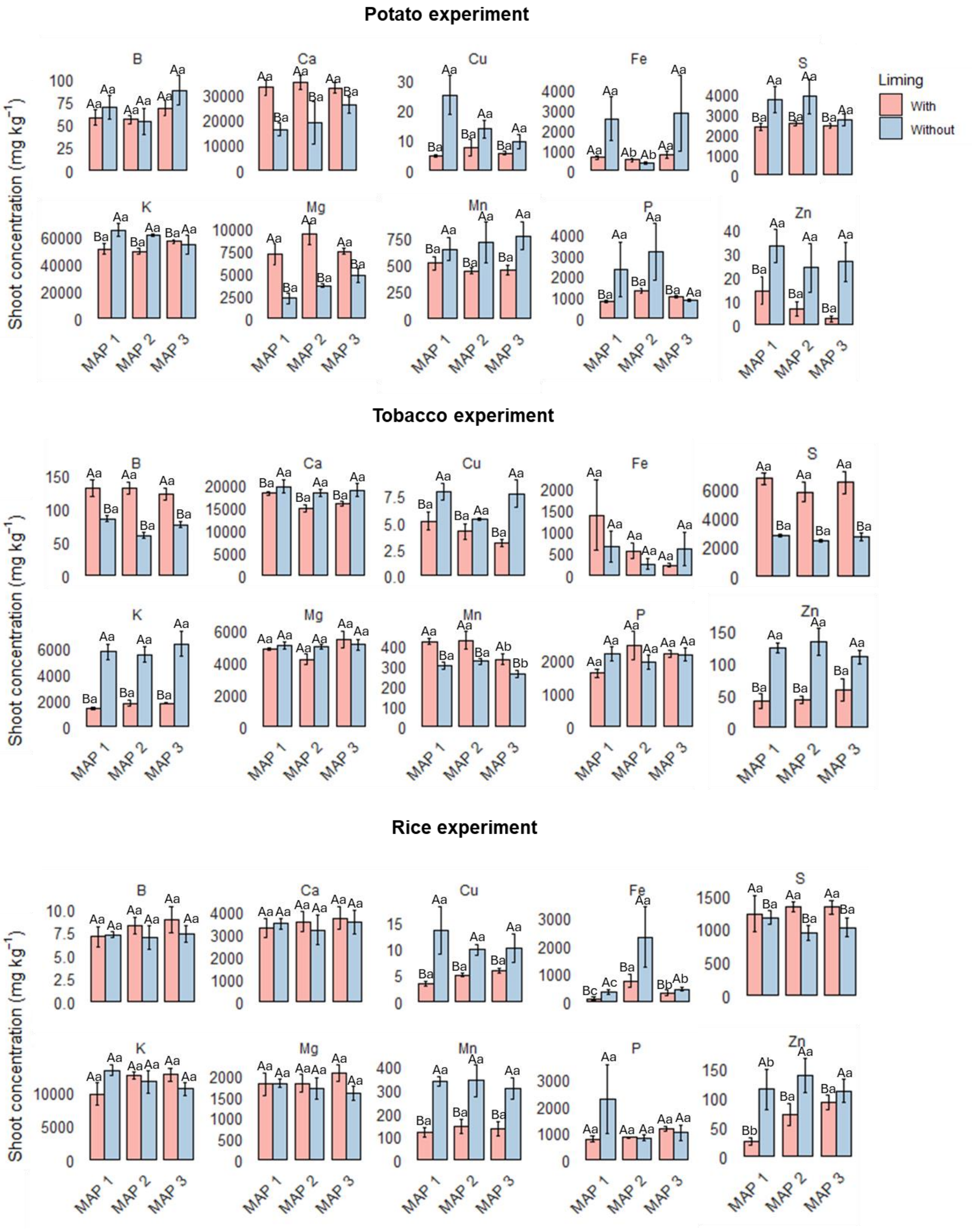

Figure 9: Macro and micronutrient concentrations in plant shoots for potato, tobacco, and rice growth, respectively, in response to liming and MAP treatments. Values are means of four replications ± standard error. Different uppercase letters represent significant differences between liming treatments and different lowercase letters indicate differences among fertilizer treatments in each liming treatment by Duncan's test ($p < 0.05$).

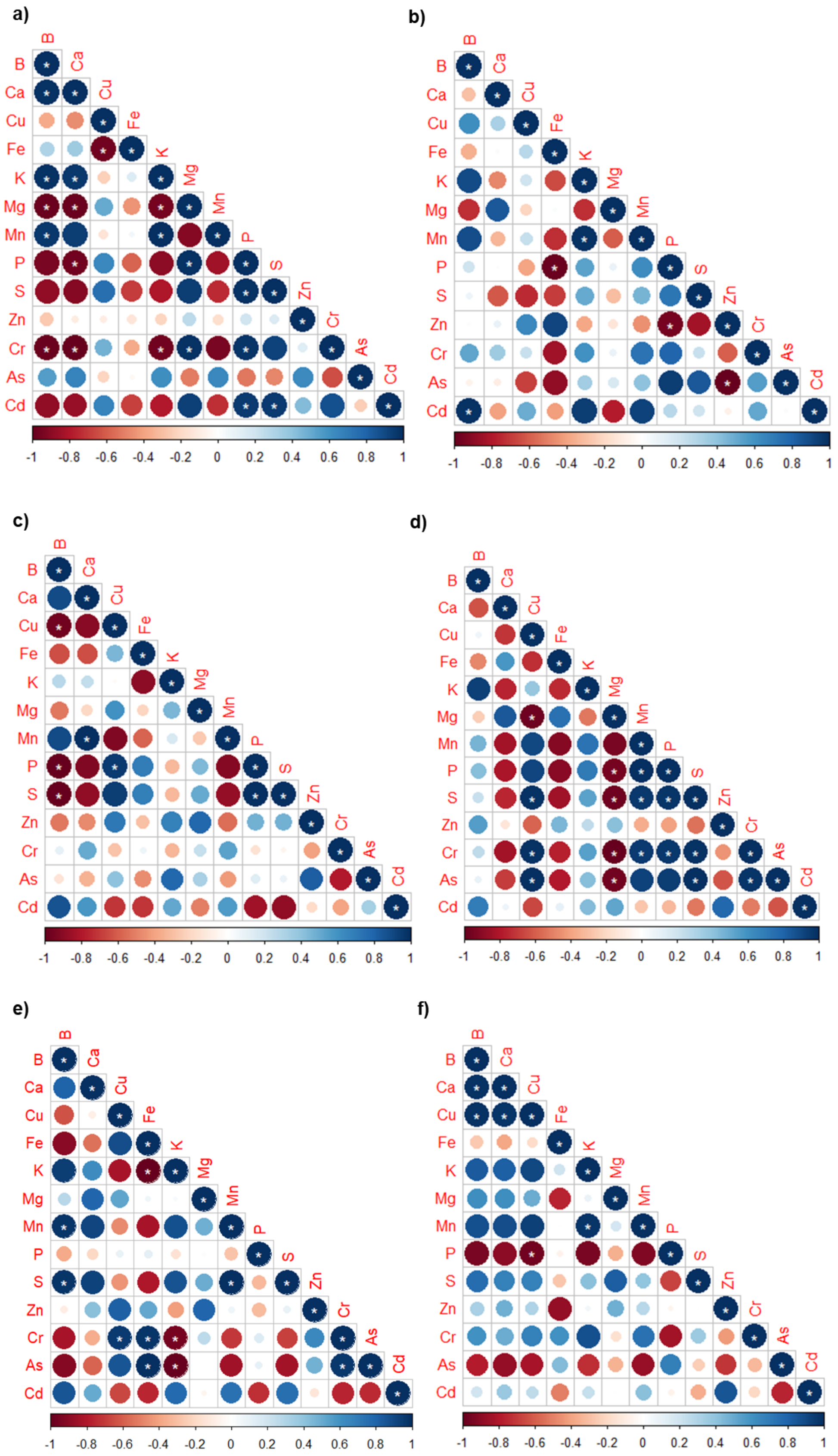

Figure 10: Correlogram matrix for elemental content in potato shoots, which were grown at MAP 1 (a, b), MAP 2 (c, d), and MAP 3 (e, f) in native pH (left) and with liming (right).

Pearson’s correlation analysis with the significance levels of P < 0.05 (white *****) by Tukey test (p <0.05).

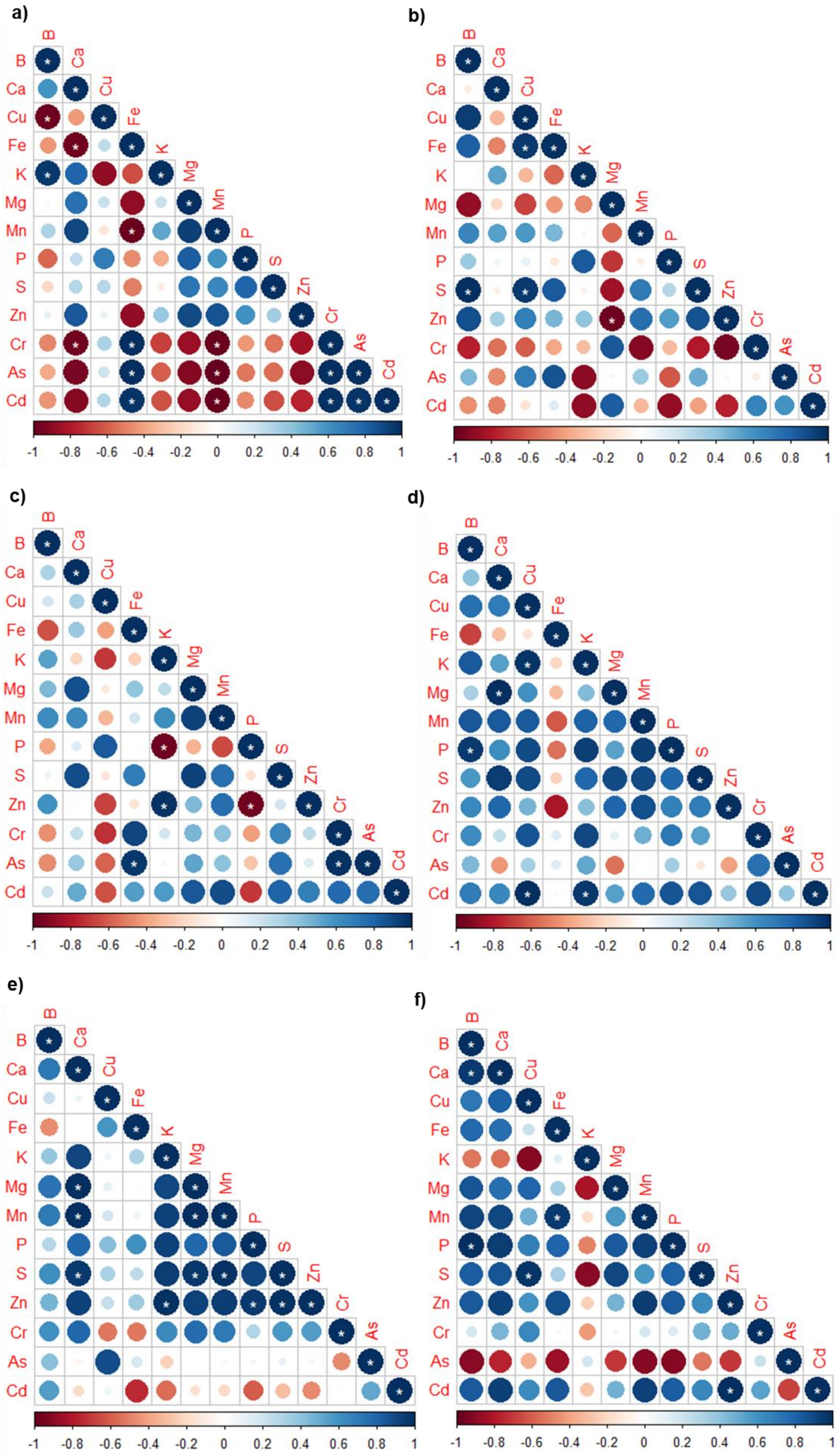

Figure 11: Correlogram matrix for elemental content in tobacco shoots, which were grown at MAP 1 (a, b), MAP 2 (c, d), and MAP 3 (e, f) in native pH (left) and with liming (right).

Pearson’s correlation analysis with the significance levels of $P < 0.05$ (white *****) by Tukey test ($p < 0.05$).

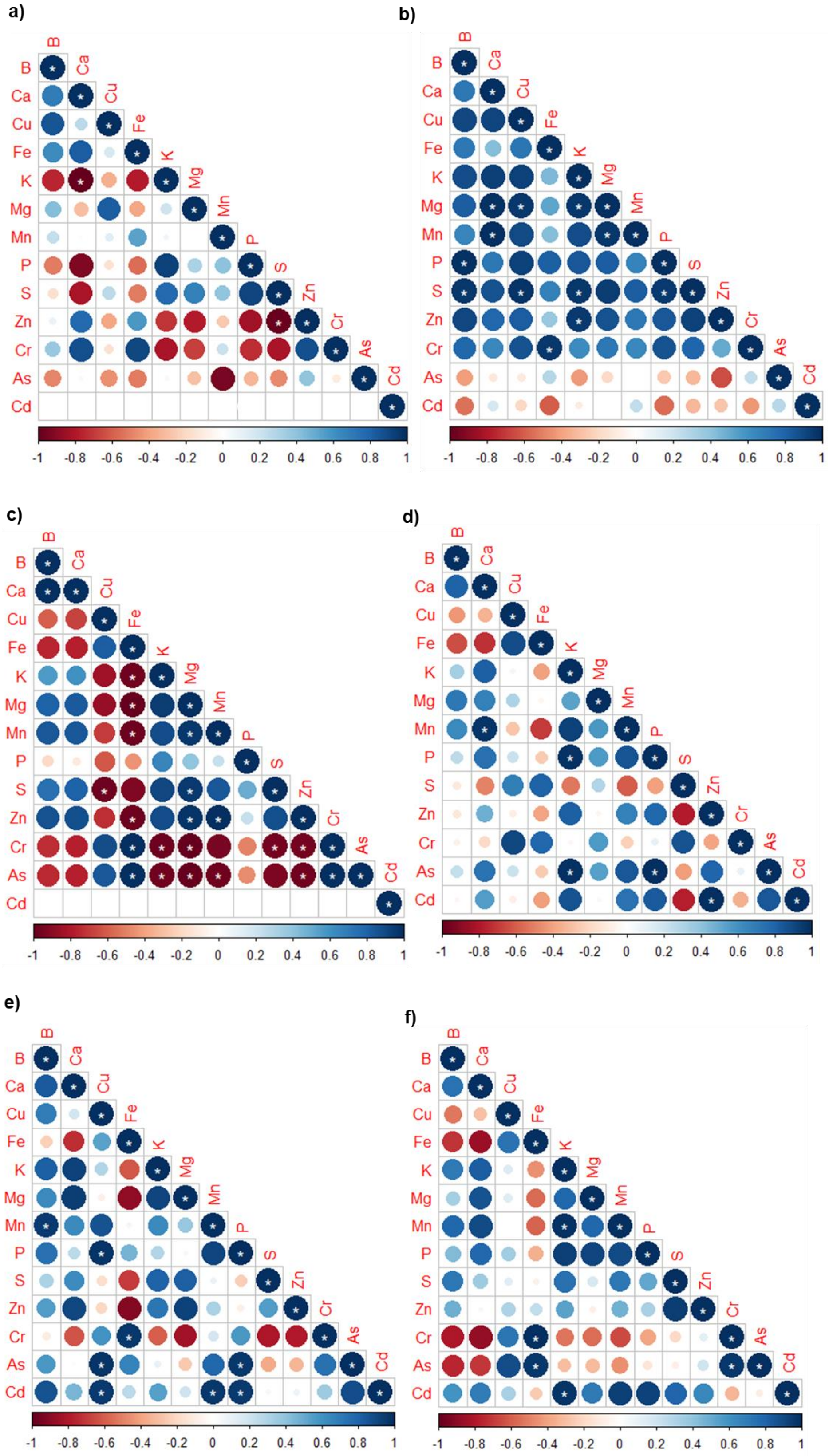

Figure 12: Correlogram matrix for elemental content in rice shoots, which were grown at MAP 1 (a, b), MAP 2 (c, d), and MAP 3 (e, f) in native pH (left) and with liming (right).

Pearson’s correlation analysis with the significance levels of $P < 0.05$ (white *****) by Tukey test ($p <0.05$).

4. Discussion

Soil pH is essential for the chemistry and fertility of soils due to the control of biogeochemical and biological processes (Neina, 2019). Solubility, mobility, and bioavailability of nutrients are driven by soil pH, which determines their translocation for plants. Besides affecting essential elements, pH also significantly affects the solubility of toxic metal(loid)s (TMs) in the soil as a main factor (Gupta et al., 2019), as observed in this study.

The bioavailability of cationic TMs (*e.g.*, Cd^{2+} and Cr^{3+}) decreases with high pH and increases with low pH (Sheoran et al., 2016), while the reverse occurs for anionic TMs (*e.g.*, arsenate - $H_2AsO_4^-$ and chromate - $HCrO_4^-$). Cadmium (Cd) dynamics in soil are particularly impacted by soil pH. Indeed, one unit of decrease in pH led to increased Cd solubility in soil ~ 4-fold (Smolders and Mertens, 2013). As shown in Figure 1, the highest values of shoot bioconcentration factor (BCF) for Cd in all three experiments were found for plants growing in most acidic soils (without liming). MAP 3 fertilizer (high Cd) was overall correlated with higher values, except for rice, where the treatments were not statistically different.

Among crops, the BCF for Cd in the lowest pH was as follows: potato > tobacco > rice. These three crops share the NRAMP protein family as a major Cd transporter (Tian et al., 2021), and although we used the same fertilizers and the same soil for the three experiments, the TMs bioaccumulation depends on several factors in the soil-plant system that differ among species and genotypes. Khan et al. (2016) also reported differential Cd bioaccumulation among lettuce > tomato > potato, but when Cd was applied with Pb the Cd concentration was decreased in lettuce and increased in potato. Furthermore, rice mechanisms to deal with Cd include decreasing translocation to shoots, and the higher Cd levels in rice are correlated with paddy soils (Rizwan et al., 2016).

Soil acidity, besides increasing TMs solubility, also becomes a challenge for plant growth and yield due to high levels of H^+, Al, Fe, Mn, Cu, Zn, and depletion in essential elements like N, P, K, Ca, and Mg (Bian et al., 2013; Wang et al., 2022). The Cd uptake and accumulation abilities vary among species, but overall, the cationic elements Ca, Cu, Fe, K, Mg, Mn, and Zn can share cell transporters with Cd in the rhizosphere and inside of plants (Lux et al, 2011; Khan et al., 2016; Naeem et al., 2019; Júnior et al., 2014; Teles et al., 2022;

Feng et al., 2017). Hence, we investigated the interaction of levels of these elements and plants treated with MAP 3 (higher Cd) and very acidic soils (without liming) for three plant species.

In the potato experiment, the Mn level in the soil with MAP3 was statistically higher than in the other two treatments (Figure 8). Conversely, in the tobacco experiment, Zn soil levels were higher for MAP 3, while Mn in the shoot decreased compared with MAP 1 and 2 (Figure 9). Both Mn and Zn share with Cd the transport by NRAMP proteins (Tian et al., 2021). As is known Zn, as a divalent cation competes with Cd by binding sites in soil and rhizosphere (Rizwan et al., 2019), and Mn also has an antagonistic interaction with Cd due to the inhibition of transport from roots to shoots (Júnior et al., 2014; Telles et al., 2022).

In this work, rice did not show differences among MAP treatments in nutrient content, while in potato and tobacco, the elements were affected differentially by higher Cd in MAP 3. A previous study reported that Mn concentration was increased in potatoes and decreased in tomato plants at the same Cd concentration, while the concentration was not altered in lettuce plants (Khan et al., 2016). These contrasting results among species may be associated with the uptake mechanisms and synergic and antagonistic effects.

Potato plants did not present alterations in primary metabolism in response to a high level of Cd in MAP 3. On the other hand, tobacco plants grown with MAP 3 in acidic soils showed a decrease in the effective quantum yield of photosystem II (ϕPSII), and coefficient quenching related to open PSII (qP) (Figure 4k, m). However, no permanent damage was indicated, and this did not affect growth or other metabolism in these plants. Interestingly, rice plants, which had the lowest BCF for Cd, show higher levels of H_2O_2 in MAP 3 treatment, followed by an increase in enzymatic activity and sugar content (Figure 5i – l, and Figure 6i – l). These metabolic responses are commonly related to Cd and other toxic metal(loid)s (Shahid et al., 2019; Xie et al., 2014; Jin et al., 2024; Li et al., 2020).

As expected, the most acidic pH decreased the growth of potato plants and lowered the tuber yield by ~ 50% (Figure 2e). This result in potato growth can be supported by the finding that the Ca and Mg content in shoots was strongly decreased (Figure 9). This result might be related to competition with high Al content in acidic soils, and similar results were related to citrus and sugar beet, which had a decrease in the level of these elements at low pH (Long et al., 2017; Wang et al., 2022).

Both Ca and Mg elements are crucial for plant growth. Calcium is a structural component of plant cells and acts as a secondary messenger and a signaling molecule for plant growth and response to environmental stress (Thor, 2019). Magnesium is a chlorophyll structure constituent (Marschner, 2011), acts as an enzymatic activator, and plays a role in photosynthesis, carbohydrate transport, and protein synthesis (Papadakis et al., 2021).

Similarly to potato plants, tobacco growth also was strongly affected by soil pH (Figure 2j - i). However, for this experiment, Ca and Mg levels were not decreased, in contrast, the Ca level increased in tobacco shoots in acidic soils. (Figure 9). Although depletion of these elements is usually related to pH decreasing, these responses seem to be dependent on experiment conditions and plant species.

Our results showed that the lowest pH induced a little decrease in the effective quantum yield of photosystem II (ϕPSII) for potato and tobacco plants (Figure 4d, k). This was followed by a decrease in qL and qP (photochemical extinction coefficients) for both species, respectively (Figure 4f, m). In citrus leaves, a pH of 2.5 led to a decrease in ϕPSII, followed by Fv/Fm, Fv'/Fm, and ETR indicating damage in the whole photosynthetic electron transport flux (Long et al., 2017). However, in this study no alterations were observed for Fv/Fm parameters, which represent the integrity of photosynthetic performance, indicating no occurrence of damage. This result suggests that the yield of PSII was affected by soil acidity, probably due to the depletion of nutrients such as Mg, Mn, and N, which are usually decreased in these acidic conditions. Specifically for tobacco plants, this result in MAP 3 treatment might be due to a combination of acidic pH and higher TMs concentration.

A little decrease in H_2O_2 content was observed in potato leaves grown in the most acidic conditions compared with limed soils, which was consistent with the highest activities of antioxidant enzymes SOD and CAT. In the rice experiment, the H_2O_2 level increased in these same conditions, followed by an increase in enzymatic activity. Similarly, in sugar beet leaves, the lower pH also increased antioxidant activities, followed by a lack of nutrients and susceptibility to uptake of toxic metals (Wang et al., 2022).

H_2O_2 is a common product of aerobic metabolism in plants and can be produced from photorespiration, electron transport chain, or redox reactions in various plant compartments (Niu and Liao, 2016). This molecule is key signaling of several physiological processes and is naturally scavenged, through non-enzymatic and enzymatic reactions. The enzymatic process involves SOD and CAT actions to convert this molecule into H_2O and O_2 (Sewelam et al., 2016). However, H_2O_2 and other ROS can be associated with oxidative stress when the production exceeds the repair capacity of the antioxidant system, leading to an unbalance in cell redox status (Niu and Liao, 2016).

Although no pattern was observed for H_2O_2 or enzymatic activity, the level of H_2O_2 in tobacco leaves was almost 20-fold higher than the levels found in potato and rice. Hence, we suggest that an oxidative stress might have occurred. Conversely, results for tobacco leaves shown in Figure 6 propose a metabolic adjustment in response to stressful conditions caused by low soil pH. This is due to the observed increase in amino acids and proline levels, while protein synthesis decreased, compared with limed soils. Enhancing enzymatic activity is a cellular response associated with tolerance for low pH, as well as an increase of osmoregulatory substances (Wang et al., 2022; Geng et al., 2021).

The sugar content in shoots for the three experiments (potato, tobacco, and rice) was reduced in response to acidic soils. This result can be related to growth and nutrition depletion under these conditions. Soluble sugars are used to sustain plant growth and play as signaling molecules regulating different genes, especially involved in photosynthesis, osmoregulatory synthesis, and carbohydrate metabolism (Eveland and Jackson, 2012).

Sugar signaling interacts with other metabolic pathways to form a complex network for regulating plant growth and development, and response to environmental conditions. Although several studies have shown that sugars accumulate under stress as a tolerance strategy, we suggested that reduction in sugars under very acidic pH might be correlated with nutrient depletion experienced by these plants, leading to a source-sink unbalance. We speculate about root exudation to cope with nutrient lack (Carvalhaes et al., 2010).

Pearson correlations were used to find correlations among elements in response to TMs levels in MAP fertilizers, soil pH, and plant species. Many variations were observed, but overall, the most often correlations involved As and Cr, and among plants, rice had fewer correlations than potato and tobacco, probably because this crop accumulated less Cd and Cr. No negative correlations were observed between Cd, As, and P, as well as no imbalance in P level was observed in response to MAP 3 (Figure 9). In general, As, Cd, and Cr were positively correlated in this study, and associated with P. This may be attributed to the use of MAP fertilizer as a source of these TMs loading to synergistic effects.

Arsenic, Cd, and Cr were often positively correlated with Cu and Fe. These correlations can be associated with synergistic effects on transporters and common translocation systems. Specifically, plants with MAP 3 (higher Cd) showed positive correlations between Cd Mn and Zn. Similar correlations were reported for rice, basil, sunflower, and potato (Feng et al., 2017; Júnior et al., 2015; Khan et al., 2016; Teles et al., 2022).

Conclusions

The crops used in this study had different mechanisms to deal with Cd bioaccumulation from MAP fertilizer under contrasting soil pHs. Overall, the toxic metal(loid) level in phosphate fertilizers did not induce damage to plant development, yet some metabolic adjustments were observed. Cadmium from MAP 3 altered concentrations of Mn and Zn for potato and tobacco experiments, and induced metabolic alterations in rice plants, like antioxidant signaling and increase of soluble sugars.

Low pH leads to nutritional unbalance in soil and plants, which reduces growth and physiological parameters in primary metabolism, such as carbohydrate content. Potato and tobacco plants had a higher potential to accumulate Cd and were more sensitive to low pH showing reductions in growth, yield, photochemical process, and carbohydrate content. To tolerate acidic pH, rice and potato plants exhibited adjustments in enzymatic activities, while tobacco plants accumulated osmoregulatory substances. The soil acidity caused an imbalance in the uptake and translocation of nutrients that contribute to growth and yield reductions. These alterations if combined with high levels of TMs may induce stress in crops.

These investigations of physiological adjustments and elemental profiles in contrasting conditions related to soil pH and levels of toxic metal(loid)s improve the understanding of the potential impacts of contaminants in P fertilizers, as well as soil amendments like liming regarding crop growth in tropical soils receiving high rates of phosphate fertilization with MAP.

Acknowledgments

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Manuscript 3: Uncovering how selenium alleviates cadmium toxicity through sulfur compounds: a comparative study on lettuce and Arabidopsis

Abstract

Cadmium (Cd) is one of the most toxic and mobile trace elements in the environment and cultivated plants are prone to accumulate high levels of this element, especially leafy vegetables and cereals. Thus, establishing and investigating mitigation mechanisms for this contaminant is a food safety issue. Selenium (Se) is an essential element for humans that has been the focus of several studies when it comes to food safety and quality, but much remains to be known about the impact of this element on plants and how it interacts with other elements. In this context, we used lettuce and *Arabidopsis* plants (WT and the *cad1-3* mutant) and evaluated the phenotype, photosynthesis, sulfur compounds, and regulatory enzymes for S assimilation and nutritional balance. As markers of Cd stress, we observed reduction in root and shoot biomass, chlorosis, browning of roots, reduction in photosynthesis, synthesis and/or accumulation of compounds related to plant defense, and changes in the nutritional balance of plants, mainly the Fe content in the leaves. Although in our work Se did not prevent Cd translocation, our results showed that Se was able to alleviate some symptoms caused by Cd in plant metabolites. In general, Se was efficient in increasing root growth, improving photosynthetic performance, increasing the content of non-protein sulfur compounds, increasing the activity of S/Se metabolism enzymes, and significantly increasing the content of S, Mo, and Fe, drastically reduced by Cd stress. The results obtained with the comparative study between the WT *Arabidopsis* genotype, and the *cad1-3* mutant showed that the Cd tolerance response pathway regulated by Se is related to the synthesis of this peptide, which in turn, is widely known as a response mechanism to Cd within the plant.

Keywords: ATPS; Cadmium; Sulfur; Phytochelatin; Food safety; Selenium.

1. INTRODUCTION

Cadmium is one of the most harmful contaminants in agricultural soils. This metal has no biological function and is extremely toxic resulting in growth reduction, inhibition of photosynthesis, and alteration of ions homeostasis (Ismael *et al.*, 2019). The Cd moves across cell membranes through essential metal transporters, like Fe, Mn, and Zn. So, by replacing these elements, Cd constrains their level in plant tissues (*e.g.,* Cd reduces Fe and Mn in the shoot) (Naem *et al.*, 2019).

Although Cd results in an unbalanced nutrition status, studies have demonstrated the importance of mineral nutrition and supplementation to mitigate cadmium toxicity in plants (Dos Santos *et al.*, 2020; Oliveira *et al.*, 2020). In this context, an element that has been extensively studied to alleviate cadmium damage in plants is selenium (Se). Selenium is an essential trace element for animals and humans with antioxidant properties, and Se in low concentrations is important for plant growth and development (Boldrin *et al.*, 2018).

Previous results have shown that selenium can decrease cadmium levels in several plant species, alleviate oxidative stress, and increase photosynthesis in plants exposed to this metal, thus improving their growth (Alves *et al.*, 2020; Gao *et al.*, 2018; Li *et al.*, 2020). Also, it has been reported that Se can decrease Cd translocation and accumulation in grains by regulating transporters of this metal, in addition to facilitating apoplastic barriers by restricting the movement of this metal within the plant (Riaz *et al.*, 2021). Cadmium reduces plant growth, and photosynthetic rate and causes oxidative damage to plants, directly or indirectly through the generation of ROS, and selenium and sulfur have been related for the alleviation of Cd toxicity and antioxidant protection from metal stress in different crop plants (Adhikari *et al.*, 2018; Alves *et al.*, 2020; Mir *et al.*, 2021; Shahid *et al.*, 2019; Wu *et al.*, 2016; Zhou *et al.*, 2020).

Selenium uptake occurs by nutrient transporters and into the plant, Se is assimilated in plastids by the metabolism of sulfur (S). Thus, Se shares all regulations regarding signaling and enzymes of S metabolism pathways. The ATP-Sulfurylase enzyme (ATPS) catalyzes the first step of primary S/Se assimilation in plants resulting in a product that will be incorporated into cysteine (Cys) and is a key-regulatory enzyme in the S/Se metabolism (Anjum *et al.*, 2015; Khan *et al.*, 2016; Takahashi *et al.*, 2011).

In addition, the final step of S/Se assimilation is catalyzed by O-acetyl serine (thiol)lyase (OASTL) to directly form Cys (Pilon-Smits; Quinn, 2010). Although this enzyme had no central role in S assimilation, its activity is correlated with S and NPT concentrations (Liang *et al.*, 2016). Cysteine is the second major component of the non-protein thiol (NPT) group found in plants and is involved in the synthesis of S-containing compounds including glutathione (GSH), phytochelatins (PCs), metallothioneins (MTs), and thioredoxins (Trx) (Khan *et al.*, 2016).

These S-compounds are known for their involvement in plant tolerance to abiotic stress and metal(loid)s' homeostasis (Anjum *et al.*, 2012; Gil *et al.*, 2012; Na; Salt, 2011; Verbruggen *et al.*, 2009). Plants exposed to Cd have shown higher activity of transcripts enzymes of sulfur metabolism, including those related to sulfate assimilation pathways, such as ATPS, resulting in increasing contents of Cys and GSH, which might lead to increased biosynthesis of PCs, lesser oxidative stress, and lesser decrease in the net photosynthetic (Gill *et al.*, 2012). Khan *et al.* (2009) also reported that an increased expression of ATPS activity has a crucial role in the maintenance of high GSH levels to the AsA-GSH cycle under oxidative stress from Cd exposure.

Although the beneficial role of Se in the mitigation of metal-induced oxidative stress has been studied, a better understanding of the action mechanisms of Se in preventing metal accumulation in plants is still needed, because the mode of action on how Se increases plant tolerance to Cd stress remains unclarified and had many variations between reports. Additionally, most studies with Se's role in Cd toxicity focus on oxidative stress and antioxidant enzymes, and there are scarce discussions regarding how non-enzymatic and sulfur compounds are affected by Se in Cd-exposed plants.

ATPS has been assumed as the rate-limiting step enabling and starting S metabolism and increased ATPS activity can provide tolerance to plants under Cd stress (Anjum *et al.*, 2015). On the other hand, no studies were found correlating the OASTL enzyme activity with Se under Cd stress. Thus, we questioned the roles of ATPS and OASTL regulation and their products in the Se alleviation of Cd toxicity.

In this context, this study aimed to investigate the Se alleviation role in Cd toxicity through the sulfur compounds. We hypothesized that ATPS and OASTL enzymes are regulated by Se under Cd toxicity to synthesize S-compounds, like phytochelatin, which is involved in Se-induced physiological adjustments in response to Cd exposure. Besides, this knowledge will also shed light on the complex network between Se and S - the fourth most relevant nutrient for plants - and contribute to agricultural management practices aiming to reduce Cd levels in major crops.

To do this, we propose the use of i) *Lactuca sativa* L., which accumulates high concentrations of Cd (Zare *et al.*, 2018), and thus, the Cd level in lettuce leaves sometimes is over European regulatory limits (Tang *et al.*, 2016); and, ii) the extensively studied model plant *Arabidopsis thaliana* L. These two species are very morphologically different, and this comparative study might provide a useful perspective for investigating Cd-Se interaction in higher plants.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

Lettuce experiment

The experiments were carried out in a greenhouse at Cornell University, Ithaca, NY, USA. Lettuce seeds (*Lactuca sativa* L.) cv. Bibb was germinated in a commercial substrate (PRO-MIX NK25) in a greenhouse in a 14h-light and 10h-dark photoperiod at 25- 28 ºC. After plant emergence, ten days after germination, seedlings were washed and grown in a tray containing ¼ strength Hoagland's nutrient solution (Hoagland; Arnon, 1938). The nutrient solution comprised 2 mM KNO_3, 1 mM $KHPO_4$, 1 mM $MgSO_4$, 1mM Ca $(NO_3)_2$, 96 µM Fe $(NO_3)_3$, 12.5 µM H_3BO_3, 1.6 µM $CuSO_4$, 0.4 µM $MnSO_4$, 0.1 µM Na_2MoO_4, and 10 µM $ZnSO_4$.

After two weeks of conditioning, the seedlings of the same size were transferred to 2L black pots containing nutrient solutions. After 2 days the control solutions were changed by solutions with six treatments, containing $CdCl_2$ and Na_2SeO_4, as follows: Control, Control + 1 µM Se, Control + 5 µM Se, 15 µM Cd, 15 µM Cd + 1 µM Se, 15 µM Cd + 5 µM Se. Preliminary tests were conducted to determine doses, and time of Cd, and Se application. One week after treatment application, 48 plants (6 treatments x 8 biological replicates) were harvested individually, and their fresh weights were recorded. Part of the shoot samples were frozen in liquid nitrogen and stored at -80 ºC for biochemical analysis, and others were dried in an oven for elemental analysis.

Arabidopsis experiments

Arabidopsis thaliana (Columbia ecotype, WT) was used for all the control experiments. The *cad1-3* mutant was obtained from the Department of Crop and Soil Sciences, Cornell University. Approximately 20 mg of surface-sterilized seeds from the wild-type (WT, Col-0 ecotype) and *cad1-3* line were germinated individually in Magenta boxes containing sterile Hoagland's hydroponic growth solution (pH 5.8) inside a growth chamber with continuous light and a temperature of 23°C. After germination, seedlings were either harvested for the control experiment or transferred to new hydroponic growth solutions supplemented with 1 µM Cd and 1 µM or 5 µM Se for 7 days of treatment. For harvesting, seedlings were rinsed with distilled water, wiped gently with paper towels, photographed, and frozen in liquid nitrogen.

2.1. Phenotype and plant biomass

Lettuce plants were measured for root and shoot height, weighed for fresh weight, photographed, and placed in an oven at 60°C to obtain dry mass. After initial measurements, the root/area ratio, and the relative growth rate (RGR) were calculated. Similarly, Arabidopsis plants were photographed, and their root length was measured to obtain the RGR. Then, were placed in an oven at 60°C to obtain dry mass for elemental analysis.

2.2. Photosynthetic parameters

Net CO_2 assimilation (A), stomatal conductance (gs), intercellular CO_2 concentration (Ci), transpiration (E), and electron transport rate (ETR) were performed in fully expanded leaves using the infrared gas analyzer model LI-6800 (LI-COR, Biosciences) equipped with a multiphase flash fluorometer chamber before plants harvesting. The atmospheric conditions during the measurement were photosynthetically active radiation (PAR), 1000 m^{-2} s^{-1}, relative humidity 65±4%, atmospheric temperature 24±2°C, and atmospheric CO_2, 400 μmol^{-1}. The relative chlorophyll content was measured before harvest using a chlorophyll meter (SPAD-502; Minolta Camera, Osaka, Japan).

2.3. Measurement of total Cd, Se, and nutrient contents by ICP

The total elements from the dried leaf tissues of *Lactuca sativa* L. were determined after acid digestion as detailed in our previous report (Silva *et al.*, 2024). The acid-digested samples were analyzed for element contents using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA, United States).

2.4. Sulfur compounds quantification

Non-protein thiol (NPT) level was determined following the method of Ellman (1959). A leaf sample (700 mg) was extracted in 3 mL of 6.67% 5′-sulfosalicylic acid. Following centrifugation, the supernatant was added at a 1:9 ratio to Ellman reagent containing 120 mM phosphate buffer (pH 7.5), 5 mM EDTA, and 0.6 mM DTNB. The reaction mixture was placed at room temperature for 15 min, and the absorbance at 412 nm was read using an Epoch™ Microplate spectrophotometer. NPT content was calculated using GSH as the standard (Sigma-Aldrich).

Glutathione (GSH) content was determined by the method of Anderson (1985). Half a gram of fresh leaf was homogenized in vessels containing 2 mL of 5% sulfosalicylic acid at 4°C. The homogenate was centrifuged at 10,000 rpm for 10 min. To an aliquot of 0.5 mL of supernatant, 600 µL of reaction buffer (0.1 M Na-phosphate, pH 7.0, 1 mM EDTA) and 40 µL of 0.15% 5, 5-Dithiolbis 2-nitrobenzoic acid (DTNB) was added and read at 412 nm after 2 min. Actual values of glutathione content were determined against the GSH standard curve.

For cysteine estimation, leaf material (500 mg FW) was extracted in 5% chilled perchloric acid. The acid filtrate was treated with equal volumes of ninhydrin and acetic acid according to the standard protocol as described by Gaitonde (1967). The absorbance at 560 nm was read and the amount of cysteine was calculated using a calibration curve.

2.5. ATPS and OASTL activity assays

ATP sulfurylase (ATPS, EC 2.7.7.4) activity was measured based on molybdate-dependent formation of pyrophosphate as described (Lappartient; Touraine, 1996). To prepare the enzyme extracts, the ground leaf samples (100 mg) were mixed with an extraction buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 10 mM EDTA, and 0.01 g/mL polyvinylpyrrolidone (PVP). The mixture was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was used for ATPS activity assay. To start the enzyme reaction, 0.1 mL of extract was added to 0.5 mL of the reaction mixture containing 80 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 2 mM Na_2ATP, and 0.032 U/mL of sulfate-free inorganic pyrophosphatase with 5 mM Na_2MoO_4 (Sigma-Aldrich). The mixtures were incubated at 37°C for 15 min. After the reaction time, the samples were subjected to determination of inorganic phosphate using a coloring reagent solution (ammonium molybdate + Malachite Green), and their absorbances at 340 nm were read using an Epoch™ Microplate spectrophotometer. The ATPS activity was determined by the phosphate standard curve.

O-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8) activity was determined according to the method of Riemenschneider *et al.* (2005). Leaf samples (100 mg) were macerated in 1 mL of chilled 20 mM Tris-HCl (pH 8) and centrifuged at 12,000 g for 10 min at 4°C. To measure OASTL activity, a reaction was carried out containing 1 mL of 100 mM Tris-HCL (pH 7.5), 5 mM OAS, 33.4 mM DTT, 5 mM Na_2S, and 50 µL of the enzyme extract. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped with 1 mL of acid ninhydrin (0.8% ninhydrin [m/v] in an HCl:HOAc solution in a 1:4 ratio). Color development occurred by boiling the samples at 100°C for 10 minutes. Then, 900 µL of 70% alcohol was added to 100 µL of the reaction to stabilize the color. The sample was read at 560 nm on a microplate reader and enzyme activity was determined using a cysteine standard curve.

2.6. Statistical analysis

The results were analyzed using the statistical software SigmaPlot 12.5 and differences between treatments were compared using the T-test at 5% probability. The graphs were performed on R (R Development Core Team, 2018).

3. RESULTS AND DISCUSSION

3.1 Growth and phenotype under Cd-Se exposure in lettuce and Arabidopsis

Phenotypic differences were observed between control and 15 µM Cd (Figure 1). The toxicity symptoms observed here were like those observed in a toxicological study with lettuce (Monteiro *et al.*, 2009). Cadmium treatment resulted in chlorosis and root browning and decreased lettuce shoots' length and fresh weight (Figure 1b, f). However, no effect was observed in shoot dry biomass (Figure 1d). For roots, Cd did not decrease length, but the fresh and dry weight was reduced by about 50% compared with the control (Figure 1a, e), thus Cd reduced the root/shoot ratio (Figure 1f).

Plant growth is a classic parameter to evaluate metal toxicity and may be associated with biochemical changes. In this study, selenium did not amend the growth reduction caused by Cd as reported for tomato plants (Alves *et al.*, 2019, 2020). However, the leaves treated with Cd + Se were greener and with fewer dark spots than only Cd-treated leaves, suggesting that although Se did not improve the plant growth, this element increased the photosynthetic pigments as was described for nickel stress in lettuce (Hawrylak-Nowak *et al.*, 2020). Plants differ regarding capacities to tolerate and metabolize Se (Santiago *et al.*, 2020). It is known that, besides experimental conditions, Se effects on Cd toxicity depend on Se and Cd forms, application, concentration, time, and plant species and genotypes (Feng *et al.*, 2013; Wang *et al.*, 2016).

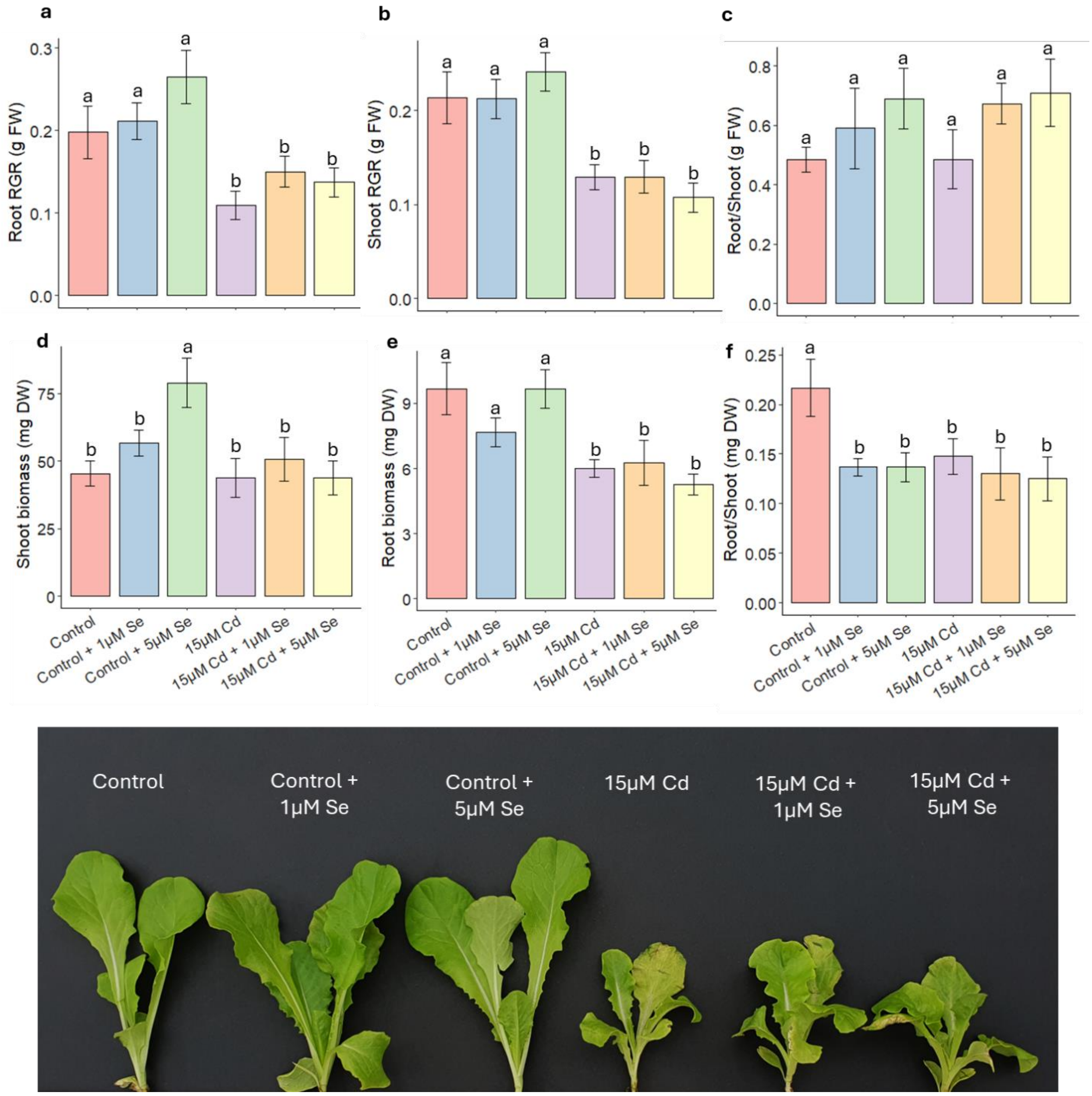

Figure 1: Relative growth rate of roots (a) and shoots (b), root/shoot ratio for fresh weight (c), dry biomass for shoot (d) and roots (e), and root/shoot ratio for dry weight (f) of lettuce seedlings subjected to 15µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at p<0.05 by T-test. The image below shows plant shoots under different treatments.

In the experiment carried out with Arabidopsis, also there were differences in the phenotype concerning the treatments and the two genotypes tested (Figure 2). Cadmium reduced plant growth, especially root length (Figure 2 a-d). However, when Se was added to the solution, root length was equivalent to control plants for the WT line (Figure 2a). Furthermore, this beneficial effect was not observed in the *cad1-3* mutant genotype, which presented more chlorosis and root browning than the WT genotype when subjected to Cd, showing greater sensitivity to Cd and less mitigating effect of Se (Figure 2b-d). The *cad1-3* mutant is a recessive and loss-off function in the Arabidopsis *AtPCS1* gene (Ha *et al*., 1999) and shows no phytochelatins (PCs) production. So, we suggested that the physiological pathway in which Se improves the root growth in Cd-exposed Arabidopsis involves PCs synthesis.

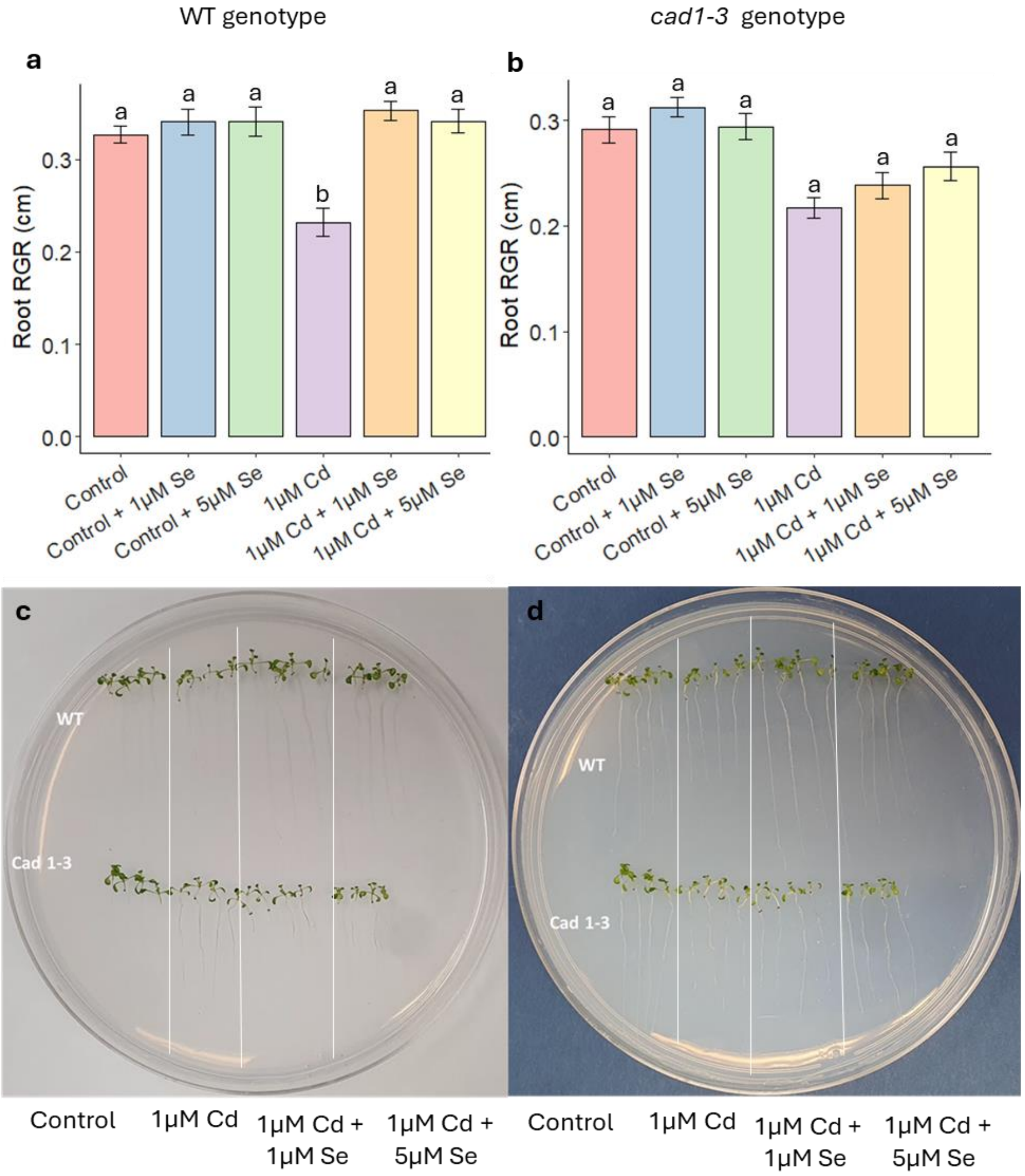

Figure 2: Root relative growth rate for WT (a) and *cad1-3* (b) lines, root darkening, and chlorosis in lines of Arabidopsis seedlings subjected to 1µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test. The image below shows plants under different treatments.

3.2 Photosynthesis parameters under Cd-Se exposure in lettuce

As observed in the phenotype, Se alleviated chlorosis in Cd-treated lettuce leaves, so we evaluated parameters related to photosynthesis (Figure 3). The net CO_2 assimilation (A), concentration intercellular of CO_2 (Ci), stomatal conductance (gs), transpiration (E), electron transport rate (ETR), and SPAD index were lower in Cd-treated plants (Figure 3a, b, c, d, e, g, i). Additionally, Cd increased water use efficiency and ETR/A, compared with the control (Figure 3f and h). Photosynthesis was increased by about 50% when 15 µM Cd and 5 µM Se were applied together (Figure 3a), followed by improvement in other photosynthetic parameters, except for ETR (Figure 3g). The WUE and ETR/A that were increased by Cd, were reduced by a control level under 5 µM of Se (Figure 3f and h).

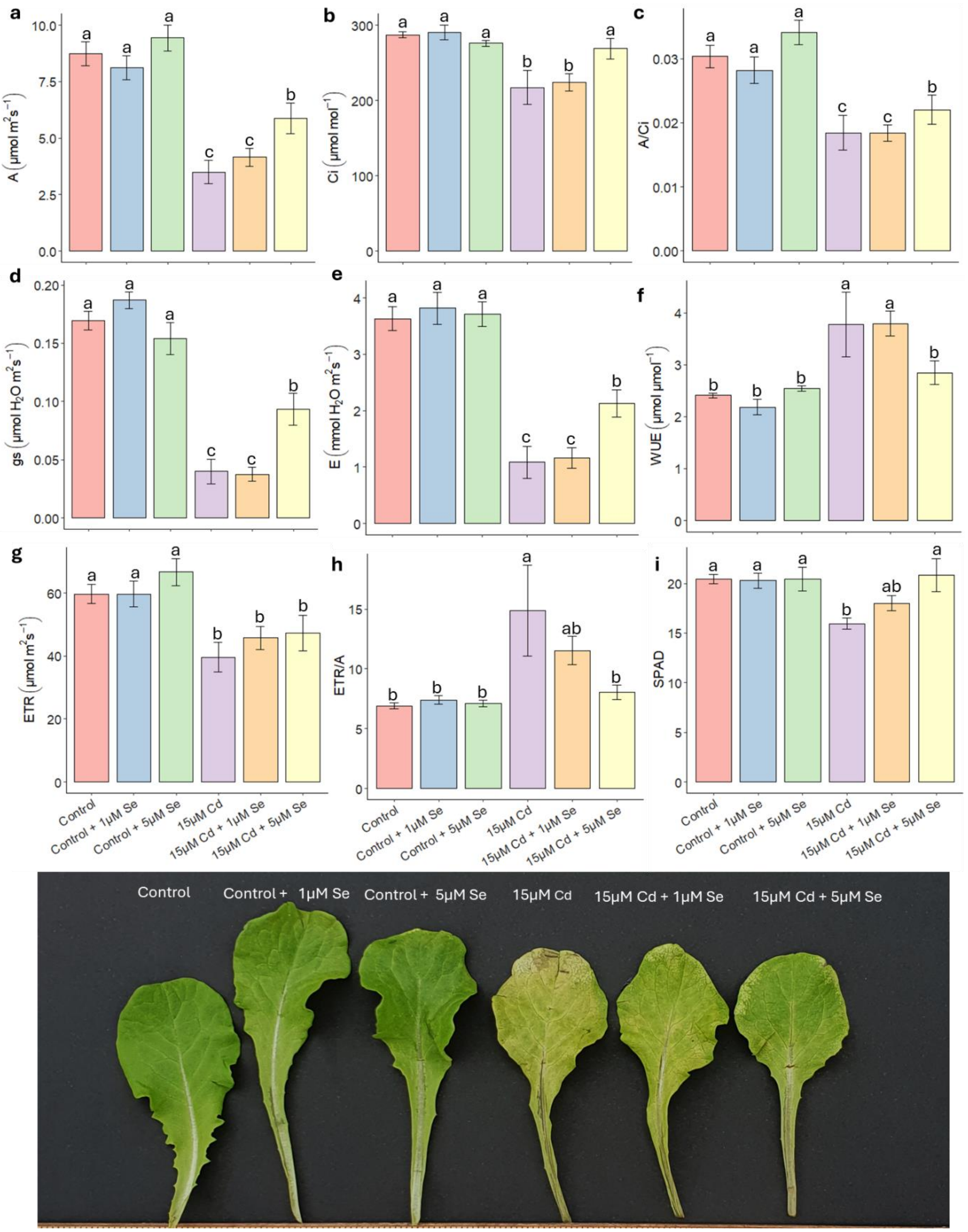

Figure 3: Photosynthesis (a), concentration intercellular of CO_2 (b), A/Ci ratio (c), stomatal conductance (d), transpiration (e), water use efficiency (f), electron transport rate (g), ETR/A ratio (h) and SPAD index (i) for lettuce seedlings subjected to 15µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test. The image below shows leaves under different treatments.

Cadmium may affect photosynthesis by damage to chloroplast ultrastructure and inhibition of chlorophyll synthesis (Wang *et al.*, 2014). This can be corroborated in this study by chlorosis and reduction in the SPAD index. As we observed in our results, cadmium may also accumulate in guard cells loading to stomatal closure (Zhang et al., 2024) and reduce CO2 conductance and transpiration. The Cd-treated plants showed higher ETR and WUE, which we can associate with the stomatal closure decreasing CO_2 assimilation and loading to energy release; as well as maintenance of intercellular CO_2 concentration (Engineer *et al.*, 2016).

Although Se application did not increase plant biomass, this element was efficient in improving the photosynthesis damages observed in this study. The effects of Se on photosynthesis Cd-damage has been reported for several plant species, *e.g.*, tobacco (Liu *et al.*, 2014), tomato (Su *et al.*, 2022), wheat (Bayat *et al.*, 2022) and rape (Zhang *et al.*, 2020).

3.3 Cd, Se, and nutrient contents under Cd-Se exposure in lettuce

The total levels of Cd, Se, macro, and micronutrients in lettuce plants were quantified. Both doses of Se applied were efficient in increasing the content of this element in the leaves (Figure 4b), as well as S concentration in the leaves (Figure 4c). Sulfur is an essential element for plants and improves growth and antioxidant systems under plant stress, including Cd stress, when was shown that S reduced Cd uptake and promoted synthesis of NPT to alleviate Cd effects in pakchoi and rice (Liang *et al.*, 2016; Zhang *et al.*, 2013b). In our study, S content in shoots was increased by Cd and Cd + Se, compared with the control, as reported by Boldrin *et al.* (2016).

Regarding Cd content, Se did not prevent Cd from being translocated to the leaves (Figure 4a). Although studies have reported a decrease in Cd uptake and translocation in the presence of Se, this element is not always effective in inhibiting Cd transport into the plant. Indeed, Alves *et al.* (2020) reported an increase in Cd levels in tomato shoots when Se was applied together, compared with Cd alone, and Se showed no effect under Cd uptake and translocation in rice (Yang *et al.*, 2019).

As mentioned in this study, PCs bind Cd into complex plants to immobilize and reduce their toxicity (Ahmad *et al.*, 2019). Cd-exposed plants tend to contain a higher percentage of Cd-sulfur ligands. Previous studies suggest that Se reacts with Cd to form Cd-Se complexes in soils (Huang *et al.*, 2018; Guo *et al.*, 2021). It is possible to speculate about the formation of Cd-Se and Cd-S complexes in this study because we observed that the concentrations of Se and S were higher in Cd-exposed plants, compared with S/Se supplementation alone, and Cd level was not decreased in lettuce shoots but the plants with Se showed some physiological adjustments.

Cadmium is known to interfere with plants' uptake and translocation of nutrients, specifically cations ions through competition by transporters (Khan *et al*., 2015). In our study, Cd affected the shoot level of some essential elements in lettuce plants (Figure 5). In opposition to the expected, Zn and Mn levels were not affected by Cd in this work. Cd-treated plants showed an increase in shoot content for B, Ca, K, and P (Figure 5a, b, e, i), while the levels of Cu, Fe, and Mo were reduced in Cd-treated plants (Figure 5c, d, g). Similar results were observed by Khan *et al*. (2016) and Júnior *et al*. (2014).

The dose of 5 µM Se was efficient in reducing the concentration of K and P which were increased due to Cd, and increasing Fe and Mo which were reduced by Cd in plants, mainly Fe. Iron in plants is a structural component of chlorophylls and a cofactor of several antioxidant enzymes (Júnior *et al*., 2014; Naem *et al*., 2019). This result about Fe level changes can be associated with chlorosis and photosynthesis parameters evaluated in this study. Thus, Se might improve photosynthesis due to alleviating the inhibition of Fe transport caused by Cd.

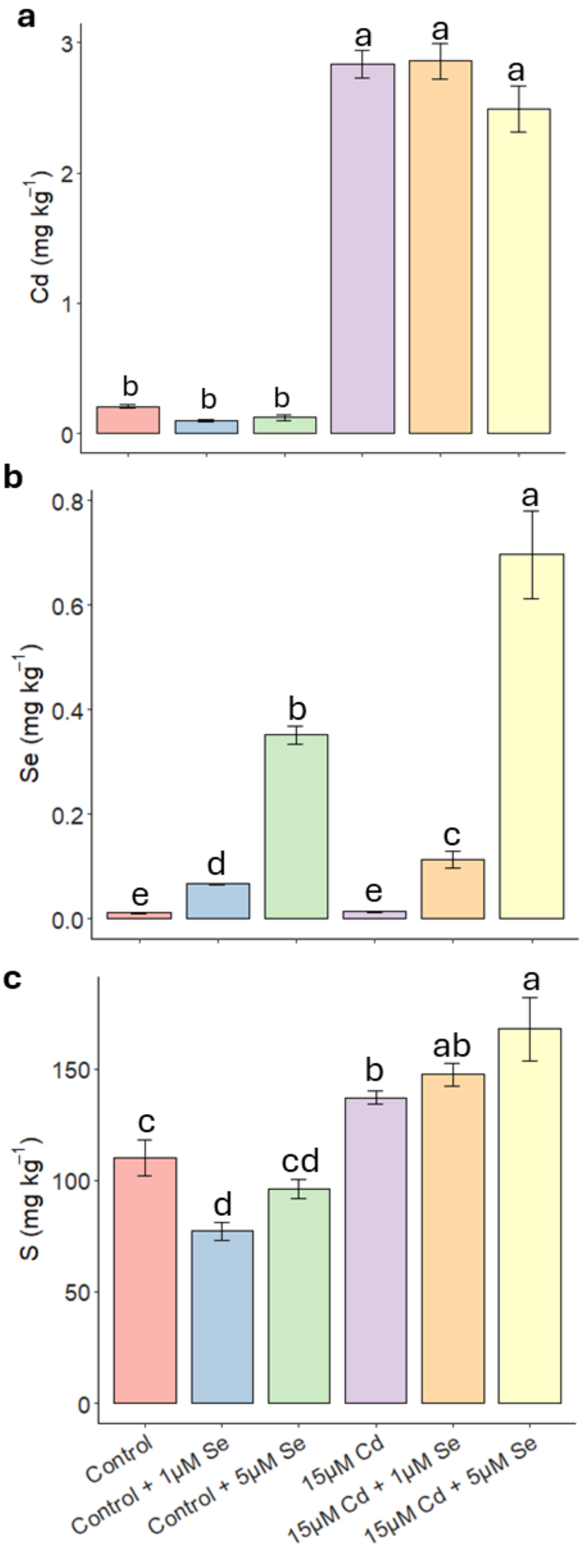

Figure 4: Total contents for Cd (a), Se (b), and S (c) for lettuce seedlings subjected to 15µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

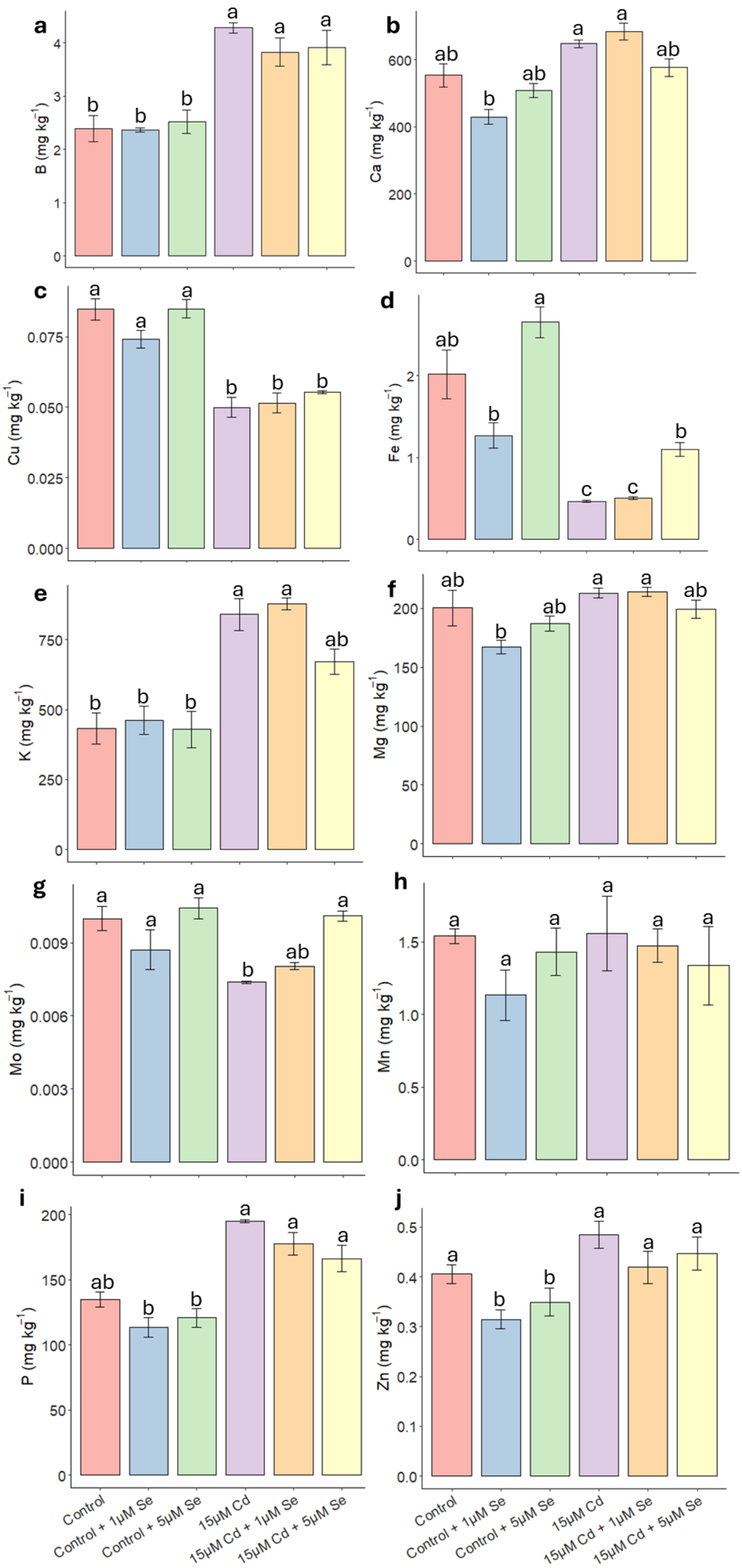

Figure 5: Total contents for Cd (a), Se (b), and S (c) for lettuce seedlings subjected to 15µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

3.4 Sulfur compounds under Cd-Se exposure in lettuce and Arabidopsis

To investigate the role of the enzyme ATP Sulfurylase, and S metabolism during Se mitigation of Cd stress in plants, we evaluated some products of the S/Se assimilation pathway that are directly regulated by this enzyme. The non-protein thiols (NPT) are mainly composed of glutathione, followed by cysteine and phytochelatin, and are non-enzymatic antioxidants essential to Cd tolerance (Zhang *et al.*, 2013). We observed an increase in the content of these compounds in the presence of Cd + Se, compared with the control (Figure 6). The content of total NPT was significantly increased when Cd and Se were applied together, in both concentrations (Figure 6a), however, cysteine and GSH contents did not reflect this increase (Figure 6b-c).

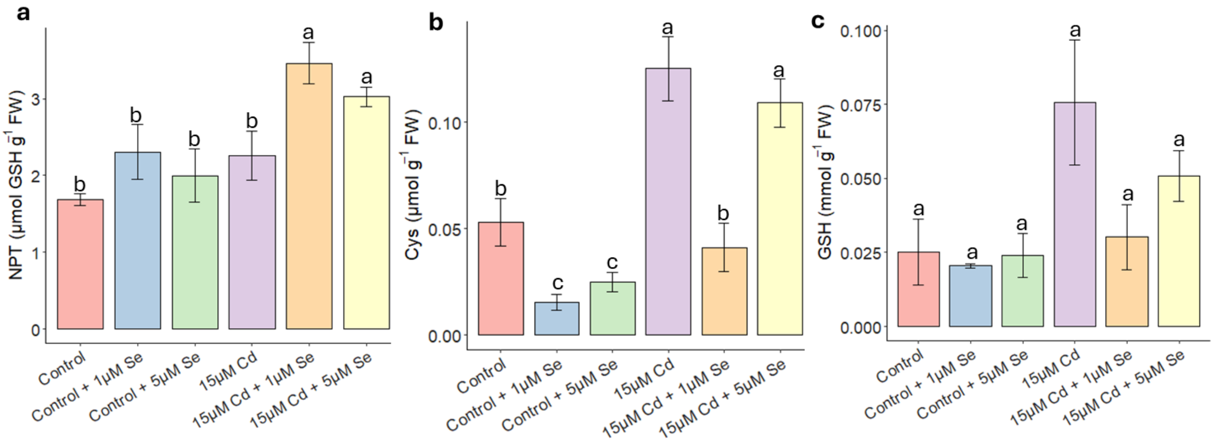

Figure 6: Non-protein thiols (a), cysteine (b), and glutathione (c) contents in lettuce seedlings subjected to 15µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

These S-compounds were quantified in Arabidopsis plants of the wild type (WT) and mutant to produce PCs (*cad1-3*) in the presence of Cd and Se (Fig 7). In the WT genotype, the dose of Cd applied did not affect the content of NPT (Figure 7a) and GSH (Figure 7e) but reduced the content of cysteine (Figure 7c), which was increased in the presence of Se, as well as NPT content. On the other hand, in mutant Arabidopsis plants (*cad1-3*), a reduction in NPT and GSH content was observed, but cysteine content was increased when Cd and Se were applied together (Figure 7b, d, f).

The accumulation of NPT is induced by Cd stress in plants (Li *et al.*, 2019; Mir *et al.*, 2022; Zhang *et al.*, 2013). Our results suggest that Se stimulates the S/Se assimilation pathway, promoting the production of non-protein thiols that help in tolerance against Cd in lettuce and WT Arabidopsis. Some authors use the difference between total NPT and GSH to estimate PCs content (Liang *et al.*, 2016). According to this, we can speculate about an accumulation of PCs in lettuce leaves exposed to 15 µM Cd + 1 µM Se. Therefore, there is a depletion of PCs synthesis in Arabidopsis mutant plants, so we can associate this with the differences in response to Cd + Se between WT and mutant lines. Opposed to WT plants, total NPT and GSH content decreased with Se application, compared with Cd applied alone.

The role of PCs in Cd detoxification through the formation of PC-Cd complexes in plants is well-established and described (Ahmad *et al.*, 2019; Ismael *et al.*, 2019). However, there is scarce literature discussing the effect of exogenous Se on PCs content in Cd-exposed plants. In cucumber, selenate decreased PCs accumulation in roots, but no effect was observed for shoots (Hawrylak-Nowak *et al.*, 2014), while in cattail and cherry tomatoes, the PCs accumulation in leaves was higher in Cd-exposed plants treated with selenite (Ren *et al.*, 2020; Su *et al.*, 2022). No studies were found with Cd-Se exposure in Arabidopsis *cad1-3* mutants.

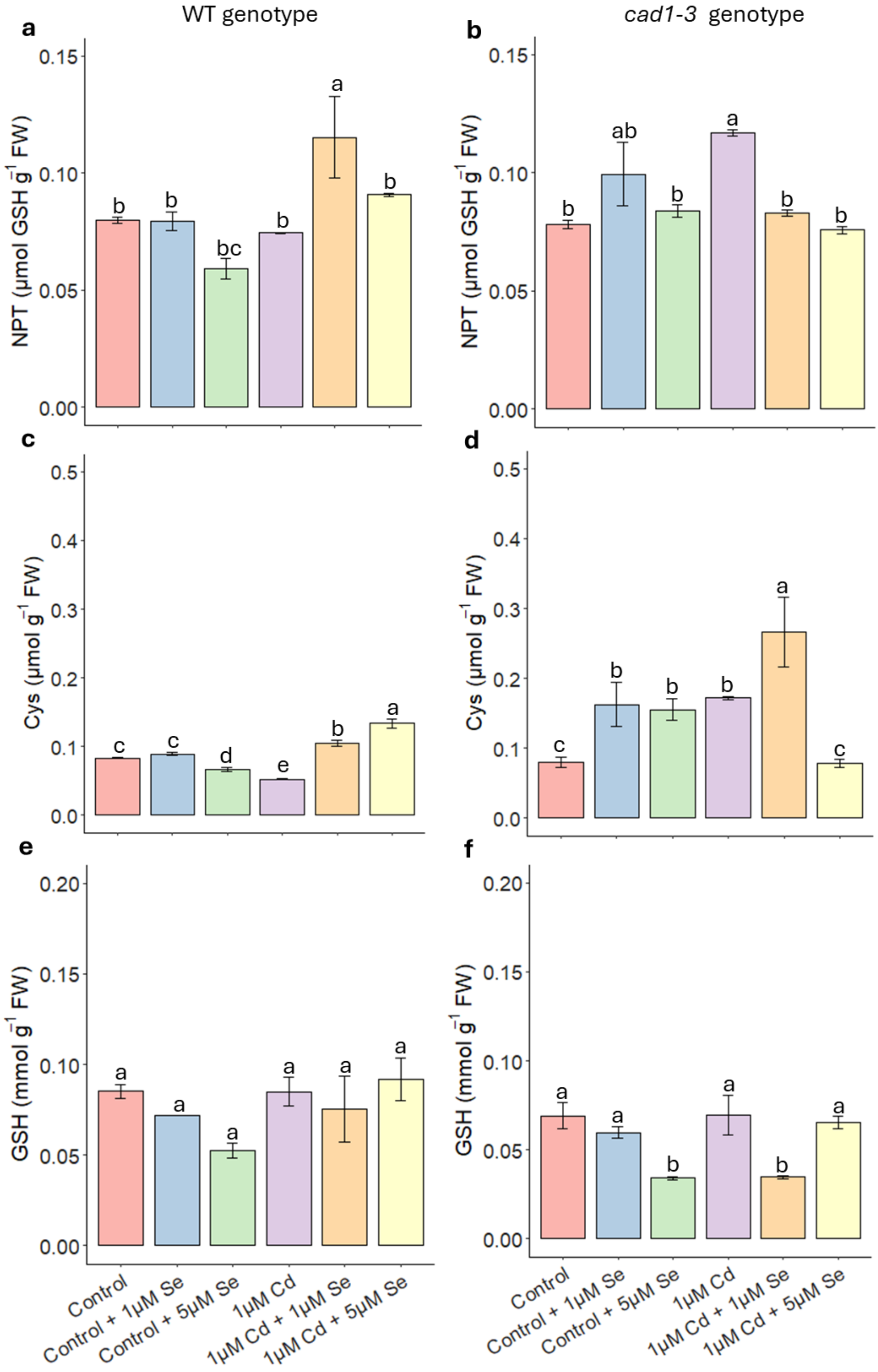

Figure 7: NPT, cysteine, and GSH contents for WT (a, c, e) and *cad1-3* (b, d, f) for Arabidopsis seedlings subjected to 1µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

3.5 Sulfur enzymes activity under Cd-Se exposure in lettuce and Arabidopsis

ATPS and OASTL are regulatory enzymes of S metabolism and are involved in the synthesis of Cys, which is a direct precursor of GSH, which is a precursor of PCs. ATPS activity enhancement was correlated with S and Se effects under Cd stress in wheat (Khan *et al.*, 2015). In our study, the activity of ATPS and OASTL were increased in Cd-treated plants compared with the control, however, when Se was supplemented at 1 µM this increase was greater (Figure 8a-b). In this same treatment, a higher level of NPT was observed in lettuce leaves (Figure 6a), while Cys and GSH levels were lower. We can suggest that these two compounds would be used for the synthesis of PCs, elevating the NPT concentration.

Regarding Arabidopsis, in *cad1-3* plants, Cd increased ATPS and decreased OASTL activities, compared with the control (Figure 9b, d), while OASTL activity was increased by Cd in the WT genotype (Figure 9c). When Se was supplemented in WT Cd-treated plants, the activity of both enzymes was enhanced, like the NPT level in this treatment. However, for *cad1-3* Cd-treated plants, only OASTL was increased and ATPS was decreased in response to Se concentrations, which can be associated with lower levels of NPT and GSH in these treatments.

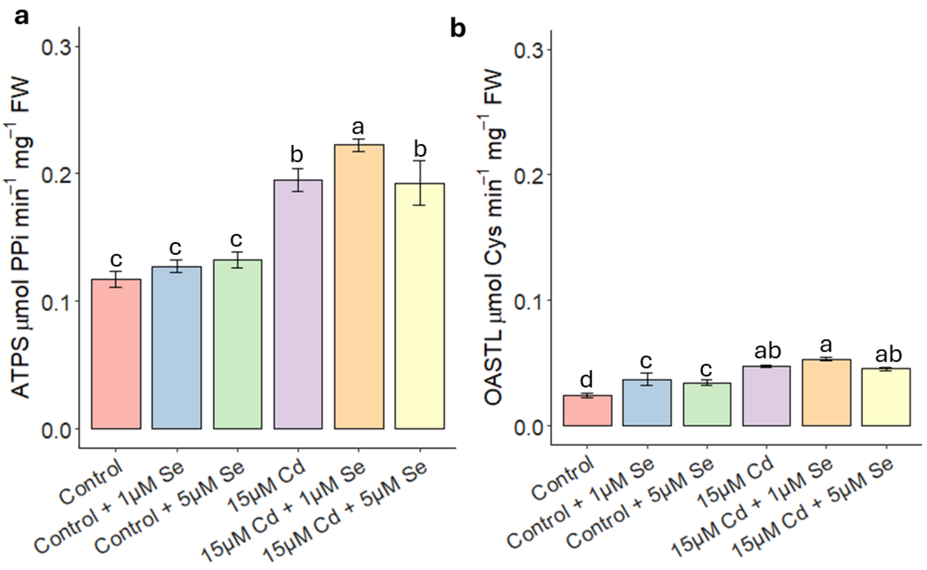

Figure 8: ATPS (a) and OASTL (b) activities in lettuce seedlings subjected to 15 µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

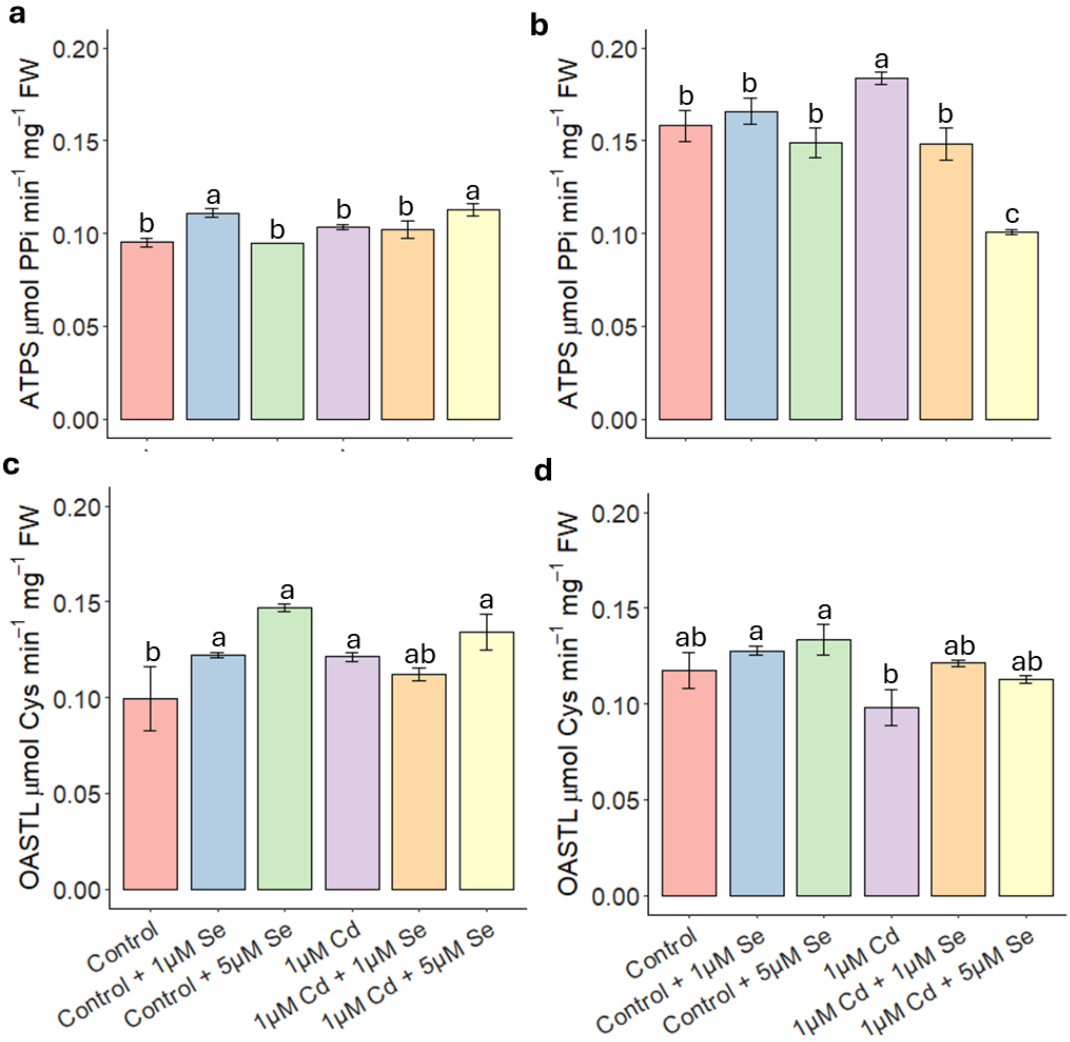

Figure 9: ATP and OASTL activity for WT (a, c) and *cad1-3* (b, d) for Arabidopsis seedlings subjected to 1µM Cd and 1 or 5 µM Se for one week. Data represents means of four biological replicates ± SE. Different letters above columns show significant differences at $p<0.05$ by T-test.

4. CONCLUSIONS

This study used two relevant species as a model to investigate the role of Se in Cd stress. We evidenced the protective role of Se on the chlorophyll status and photosynthesis of lettuce plants. The plants evaluated in this study showed different adjustments in sulfur compounds. Selenium stimulated sulfur transport in plants, as well as enzyme activities ATPS and OASTL to increase non-protein thiol accumulation. The *cad1-3* mutant showed no growth improvement, and no non-protein thiols increase due to Se application, suggesting that phytochelatins are involved in Se alleviating role in Cd toxicity.

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THIRD PART

FINAL CONSIDERATIONS

Our findings showed that phosphate fertilizers with high levels of Cd led to Cd accumulation in soils and plant tissues. In this study, the metal(loid)s concentrations did not represent human health risk, yet using fertilizers with high Cd may result in food chain contamination over time. A low pH resulted in increasing metal(loid)s concentration in soil, soil solution, xylem sap, and plant shoots and tubers.

Regarding physiological parameters, the metal(loid)s level in plants from P fertilizers did not induce growth damage. However, the different plant species - potato, tobacco, and rice - had different metabolic adjustments to deal with these elements exposure. Changes in Mn and Zn levels, antioxidant enzymes, and macromolecule content were observed in response to Cd levels in MAP 3. Again, a low pH reduced plant growth and yield and induced biochemical and nutritional alterations. Cadmium was the element most accumulated and translocated in plants. Potato and tobacco were the plant species that accumulated the greatest amount of Cd from P fertilizers.

New insights into S-compounds and enzymes in the presence of Cd and Se together were provided. The activities of enzymes ATPS and OASTL and NPT content were positively correlated with the Se mitigation effect in Cd-exposed plants. The use of *cad1-3* Arabidopsis mutant to study Cd-Se interaction suggested the involvement of PCs in Se alleviating Cd toxicity in plants.