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Selenium in soil-plant-animal systems and its essential role for human health

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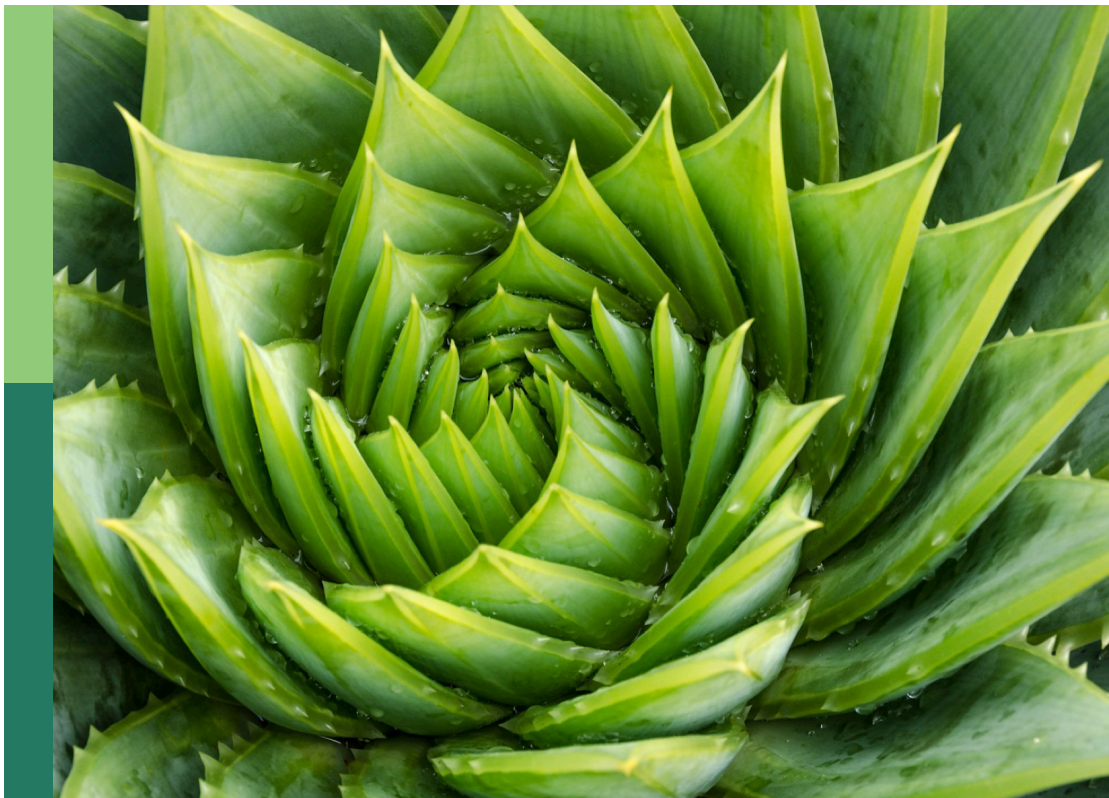

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Selenium in soil-plant-animal systems and its essential role for human health

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Editorial: Selenium in soil-plant-animal systems and its essential role for human health

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Editorial on the Research Topic
Selenium in soil-plant-animal systems and its essential role for human health

With approximate 1 billion people facing some degrees of selenium (Se) deficiency worldwide, it is imperative that the Se community work together and share the latest knowledge on various inter-related aspects of Se in supporting and protecting animal, human, and ecosystem health. In collaboration with Frontiers of Plant Science, Frontiers in Nutrition, and Frontiers in Veterinary Science, this Research Topic entitled *Selenium in soil-plant-animal systems and its essential role for human health* published original research reports and critical reviews representing different but interrelated research disciplines involving physiochemical and biological behaviors of Se within the larger foundation topics of agricultural soil, bioavailability, plant uptake, physiological responses, genetics, molecular biology, microbial communities, Se-biofortification strategies, and animal health.

Selenium is unevenly distributed in the soil, which has consequently resulted in soil Se deficiency issues and further low Se dietary intake in many parts of the world. To increase Se intake by consumption of Se-enriched plant- and animal-derived food products, we need to better understand and identify those effective strategies for Se delivery though agricultural production systems in different geographical regions. Enhancing bioavailable Se in soil will not only increase Se accumulation in crops but also result in the accumulation of specific bioactive Se compounds in food products. Importantly, the true value of successfully increasing Se concentrations in plant- and animal-based products could be highly determined by the fractionation and the speciation of Se, such as seleno amino acids or selenoproteins in Se-biofortified food products. The different Se compounds accumulated in plant tissues would further determine their bio-accessibility and the absorption of Se through human digestion systems. Similarly, animal health and reproduction are also very much dependent on the bioavailability and the absorption of Se from feeding materials. The adequate intake of Se from the feeding materials or using supplementary Se significantly affect animal's vital physiological functions that are related to reproduction or pregnancy health, and their auto-immune functions.

In this Research Topic, 15 high-quality research papers that addressed various topics or faces of Se research, ranging from the biogeochemical cycling of Se to cellular and molecular processes that elucidate mechanistic functions of Se in human and animal health. Inorganic and organic Se transformations and physio-chemical properties of soils could all ultimately determine soil Se bioavailability for plant uptake and accumulation. Both the concentration and the speciation of Se in soil could affect the Se content and the Se status of crops, particularly in edible plant materials. Recent studies demonstrated that biofortification as an agronomic-based strategy can be utilized to mitigate a low transfer of Se and other nutrients from soil into the food chain and produce Se-enriched food products, which helps increasing dietary Se intake throughout Se-deficient susceptible regions of the world.

Agronomic Se biofortification has been commonly practiced by adding Se-amended fertilizers to the soil. In Brazil, soybean is a potential major crop for biofortification. In "*Se biofortification of soybean genotypes in a tropical soil via Se-enriched phosphate fertilizers*", Silva et al. showed that the application of Se-amended phosphate fertilizers could be an effective method to deliver Se to the crop. Adding Se to the commonly used fertilizers could also be challenging due to the soil Se concentration baselines, soil types, redox protentional, pH, and soil microbial or invertebrate communities. Song et al. indicated in "*Selenium effect threshold for soil nematodes under rice biofortification"* that, with the application of selenite for rice biofortification, higher concentrations of soil Se can negatively affect soil nematodes, suggesting that the presence of soil nematodes could be used as an effective bioindicator for the soil environmental changes related to Se content. In addition to the uptake of inorganic Se, plants can also absorb Se *via* organic Se application, as shown in "*Uptake and translocation mechanisms of different forms of organic Se in rice"* by Wang, Q. et al. This rice study provides important insights into the mechanisms underlying the uptake and translocation of organic Se, especially selenomethionine (SeMet), in plants. As an alternative to soil applied Se, foliar application has been used to apply Se to plants. Schiavon et al. indicated that *"Foliar Se fertilization alters the content of dietary phytochemicals in two rocket species*," while Wang, M. et al. further outlined the differences between soil and foliar Se applications in a paper entitled "*Soil and foliar Se application: Impact on accumulation, speciation, and bio accessibility of Se in wheat*". In addition to foliar Se application, Malka et al. evaluated potential interactions between Se and Zn in foliar application, and indicated that "*Separate foliar sodium selenate and zinc oxide application enhances Se but not Zn accumulation in pea seeds".* Foliar application of Se may additionally influence plant metabolism, as well as increasing Se content in plant tissues, as shown by de Sousa et al. in "*Selenium enhances chilling stress tolerance in coffee species by modulating nutrient, carbohydrates, and amino acids context"*, and demonstrated that foliar Se application improved coffee plants' ability to tolerate chilling stress.

To produce Se-enriched agricultural products, the biofortification strategy can also be practiced in regions where there are naturally high levels of Se in the soil and/or in irrigation waters, as demonstrated by Banuelos et al. in "*Salsola soda (agretti) as a Se biofortification crop grown under high saline and boron conditions.*" Under field conditions Se-biofortified *Salsola soda* was produced with poor quality waters containing high levels of Se. Careful attention must, however, be paid in regions where Se-biofortified crops are grown in naturally Se-rich soils or with poor quality waters because of the potential presence of toxic metals in the environment. In "*Prediction models for monitoring Se and its associated heavy-metal accumulation in four kinds of acro-foods in seleniferous area"*, Jiao et al. demonstrated that models can be used to effectively predict toxic metal accumulation in Se-enriched foods in those concerned regions.

Se-enriched food products can increase Se intake and promote human health with absorption of plant tissue containing different Se compounds including seleno-amino acids. Earlier studies have clearly demonstrated the important role of Se in plant and animal physiological processes and functions. Hu et al. reviewed the importance of "S*eleno-amino acids in vegetables*", a review of their forms and metabolism and thereby affect protein structures, functional properties and antioxidant capacity in newly-germinated Se-enriched soybeans. Relatedly, Huang et al. also reported in "*Selenium biofortification of soybean sprouts: effects of Se enrichment on proteins, protein structure, and functional properties"* that Se-biofortified seeds also contain proteins whose quality has also been influenced by Se content. In addition, Li et al. evaluated "*The use of selenium for controlling plant fungal diseases and insect pests*", indicating that Se improves the plant resistance to fungal diseases.

Excessive low or high Se in soil and consequently Se concentrations in animal feeds can pose health and reproduction risks for animals. Animal-based food products for human consumption are an excellent source of dietary Se intake for the human population. Thus, safely providing Se biofortified feed materials to animals would result in increased Se concentrations in animal-based food products for humans. Hall et al. discussed Se biofortification through forages raised for livestock feed in "*Impact of selenium biofortification on production characteristics of forages grown following standard management practices in Oregon*," demonstrating that foliar selenate treatment increased forage Se concentrations in a dose-dependent manner, and that coupling Se amendment with standard fertilization practices promoted forage growth and forage Se concentrations. In cases of low soil Se, providing sulfur fertilization could reduce forage Se and potentially alter Se supply to livestock consuming those forages.

A major determinant of livestock production, health, and well-being is effective and efficient reproductive process that lead to healthy offspring. Dahlen et al. reviewed the role of Se in male and female reproductive process and the impacts of maternal dietary Se on offspring outcomes in ruminants in their paper "*Selenium supplementation and pregnancy outcomes*." The scientific evidence indicates that Se plays a major role in both male and female reproductive processes and, therefore, as a micronutrient, Se is instrumental to ensure successful animal reproductive efficiency. Increasing the maternal supply of Se alters offspring outcomes in ways that are typical of developmental programming; thereby implying that Se supply to the mother can have significant effects into the next generation of livestock. In animals, mitochondrial function is essential to bioenergetics and consequently life functions. Clearly, the role of Se in antioxidants plays a role in normal cellular metabolism and consequently whole animal health,

production, and wellbeing. In addition, Se appears to have a role in mitochondrial function besides through antioxidants. Wesolowski et al. reviewed the non-antioxidant the roles of Se in mitochondrial function in "*Beyond antioxidants: Selenium and skeletal muscle mitochondria*." The review paper demonstrates our emerging understanding of the role of Se in skeletal muscle mitochondrial function beyond the traditional constructs of antioxidants, and further highlights the importance of a greater understanding of Se in mitochondrial function and energetics.

Selenium is one of the most influential natural-occurring micronutrient elements for living systems. Recognizing selenium's impact on a multitude of processes in nature requires multi-disciplinary research on Se absorption, chemical transformation, and biochemical and physiological metabolisms in soil-plant-animal systems that can help us develop and implement effective strategies to mitigate public health impacts or concerns of Se deficiencies in the world. In this Research Topic, with different contributions from original research to critical reviews, some of the most influential researchers have provided their latest research findings and demonstrated significant advances in the field concerned with Se in food chains and its effects on human and animal health.

Author contributions

All authors (GB, Z-QL, and JC) contributed to the article through writing, reviewing and editing and have approved the submitted version.

Conflict of interest

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

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Seleno-Amino Acids in Vegetables: A Review of Their Forms and Metabolism

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Seleno-amino acids are safe, health-promoting compounds for humans. Numerous studies have focused on the forms and metabolism of seleno-amino acids in vegetables. Based on research progress on seleno-amino acids, we provide insights into the production of selenium-enriched vegetables with high seleno-amino acids contents. To ensure safe and effective intake of selenium, several issues need to be addressed, including (1) how to improve the accumulation of seleno-amino acids and (2) how to control the total selenium and seleno-amino acids contents in vegetables. The combined use of plant factories with artificial lighting and multiple analytical technologies may help to resolve these issues. Moreover, we propose a Precise Control of Selenium Content production system, which has the potential to produce vegetables with specified amounts of selenium and high proportions of seleno-amino acids.

Keywords: plant factory, precise control, selenium metabolism, seleno-amino acids, vegetables, mushrooms

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INTRODUCTION

Selenium (Se) is an essential trace element for human health. For adults (≥18 years), the recommended nutrient intake (RNI) of Se for both genders in Chinese populations is 60 μg per day (National Health Commission, 2018). Appropriate Se supplementation has been reported to exert anti-viral effects, reduce the levels of thyroid autoantibodies, and decrease the risk of cardiovascular disease, type 2 diabetes, and Keshan and Kashin-Beck diseases (Stranges et al., 2007; Rees et al., 2013; Wichman et al., 2016; Liu et al., 2018; Muzembo et al., 2019; Zhang et al., 2019a). However, 500 to 1,000 million people worldwide consume less or more than the recommended levels of Se (Shreenath et al., 2018). Both excessive and deficient intake of Se are associated with health risks. Dietary deficiency of Se in humans is associated with an increased risk of death, hypoimmunity, and cognitive decline (Rayman, 2012), while excess Se supplementation may cause toxicity as Se is involved in the generation of reactive oxygen species and oxidation of thiol compounds, which can lead to oxidative damage in cells (Rayman et al., 2018; Pyrzynska and Sentkowska, 2021). Therefore, a suitable dietary source of Se supplementation containing appropriate levels of this element could be beneficial for human health.

The main sources of Se in the diet are meats and cereals, which contribute more than 50% of the total dietary Se intake in the British and Chinese populations (Gao et al., 2011; Rayman, 2012; Yu et al., 2015). Vegetables and fruits only contribute around 7% of the total dietary Se intake in the British and Chinese populations (Rayman, 2012; Yu et al., 2015). Indeed, vegetables are recognized as relatively weak sources of dietary Se, as they generally contain less than 0.1 $\mu g\,g^{-1}$ fresh matter (FM) of Se (Rayman, 2012). However, some species of vegetables are Se accumulators, such as *Brassicaceae* vegetables, garlic, and onions (Finley, 2005). For instance, garlic can accumulate more than 1,300 $\mu g\,g^{-1}$ dry matter (DM) of Se, with 73% in the form of γ-glutamyl-Se-methylselenocysteine (γ-Glu-MeSeCys; Ip et al., 2000). Considering their relatively high consumption, rapid growth (less than 30 days for harvest of leafy greens), and ability to accumulate Se, vegetables hold great potential as Se-fortified sources for dietary Se intake.

Seleno-amino acids (Se-AAs) are organic forms of Se and are thus thought to be ideal chemical forms for Se supplementation. Organic forms of Se have been reported to be lower in toxicity compared to inorganic Se. Vinceti et al. (2017a) reported that consumption of approximately 260 μg per day organic Se led to toxic effects; the corresponding value for inorganic Se was 16 μg per day for humans. Vinceti et al. (2017b, 2018) reported that overexposure to inorganic Se was associated with Alzheimer's dementia, neurodegenerative diseases, amyotrophic lateral sclerosis, and Parkinson's disease. Moreover, dietary organic Se has high bioavailability because most of them can reach the systemic circulation from the gastrointestinal tract and can promote its action in the exposed organism. Previous studies reported that 70–90% of Se in Se-enriched plant foods could be transformed into organic forms and distribute and function in human organs and tissues (Pyrzynska and Sentkowska, 2021). Among the various Se-AAs, selenomethionine (SeMet) has the highest bioavailability of more than 90%, which is 1.5 times higher than that of selenite (Burk and Hill, 2015; Xie et al., 2021). *In vitro* simulated gastrointestinal digestion studies suggested SeMet is the major form of bioaccessible Se released from food matrices (Bhatia et al., 2013; Wang et al., 2013; do Nascimento da Silva et al., 2017). Overall, due their abundance in vegetables, low toxicity, and high bioavailability, Se-AAs are thought to be valuable forms of dietary Se supplements. In this regard, it is of interest to develop Se-enriched vegetables with high-Se-AA contents to ensure safe, sufficient intake of Se.

In this review, we critically explore the literature related to the forms of Se-AAs and Se concentrations of different vegetables, the factors that affect Se speciation, and the metabolism of Se-AAs in plant species. This comprehensive review may aid the innovation and development of Se-enriched vegetables with high-Se-AA contents in agricultural practice.

VEGETABLES AS DIETARY SUPPLEMENTS OF Se

Se-AA Forms and Concentrations in Different Species of Vegetables

Vegetables can biotransform inorganic Se into Se-AAs, such as SeMet, selenocysteine (SeCys), Se-methylselenocysteine (MeSeCys), and γ-Glu-MeSeCys (**Figure 1**). These Se-AAs can be almost completely absorbed by human organs and are beneficial for human health.

We reviewed the concentrations of total Se and the main Se-AAs in the edible parts of vegetables and mushrooms biofortified with Se (**Table 1**). The main Se-AAs in vegetables exhibit species-specific patterns, regardless of the Se source and application method. Vegetables belonging to the *Brassicaceae* and *Liliaceae* families predominantly accumulate MeSeCys,

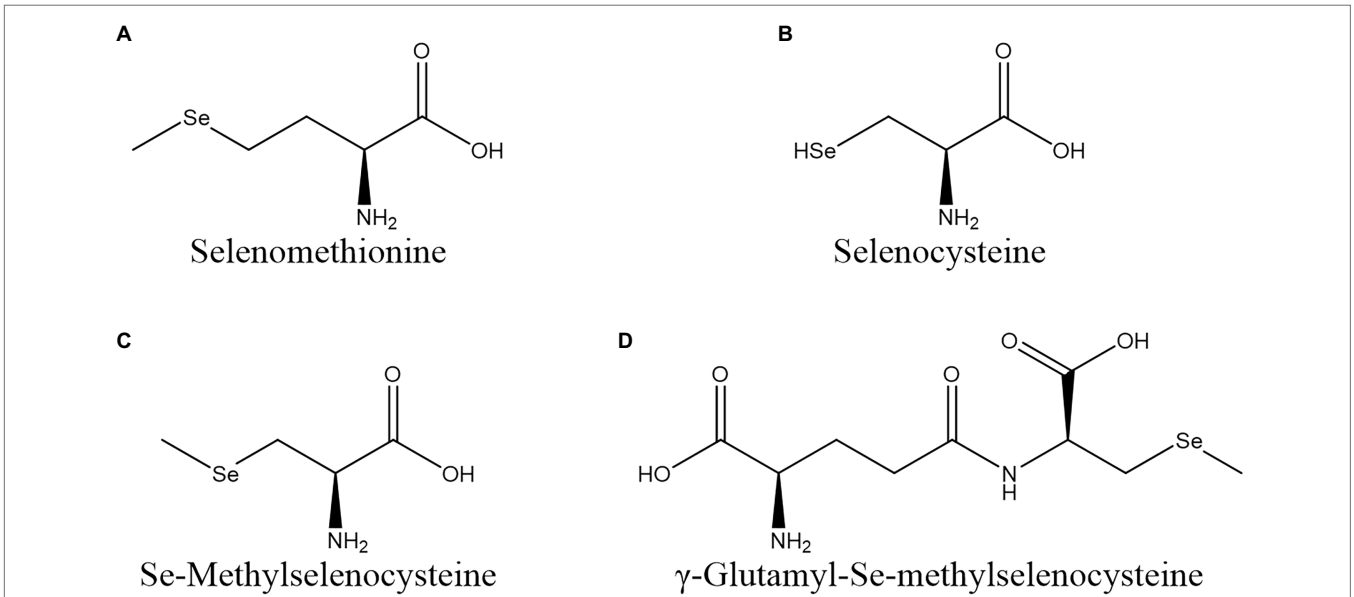

FIGURE 1 | Structures of seleno-amino acids commonly found in vegetables. **(A)** SeMet; **(B)** SeCys; **(C)** MeSeCys; and **(D)** γ-Glu-MeSeCys.

TABLE 1 | Concentrations of total selenium and the main seleno-amino acids in the edible parts of vegetables and mushrooms biofortified with selenium.

Family	Common name	Se source and dose	Application method	Total Se concentration ($\mu g\,g^{-1}$)	Main Se-AAs and concentration ($\mu g\,g^{-1}$)	Reference
Agaricaceae	Mushroom	NG	NG	9.15, DM	SeCys, 5.73, DM	Hu et al., 2021b
Agaricaceae	Mushroom	Nanoparticles, 10 $\mu g\,g^{-1}$ substrate	Substrate application	About 10, DM	SeMet, 2.01, DM	Hu et al., 2021a
Agaricaceae	Mushroom	Selenite or selenate, ≤5 $\mu g\,g^{-1}$ substrate	Substrate application	5.32 or 3.36, DM	SeMet, NG	Zhou et al., 2018
Agaricaceae	Mushroom	Selenite, 6.4 μM	Substrate application	23.1–31.0, DM	SeMet, 17.1–23.1, DM	Milovanovic et al., 2019
Agaricaceae	Mushroom	Selenite or selenate, 1.2 mM or 26.5 μM	Substrate application	111.8 or 45, DM	SeMet, 55.1 or 24.3, DM	Hu et al., 2020
Agaricaceae	Mushroom	Selenite, 5 $\mu g\,g^{-1}$ substrate	Substrate application	59.6, DM	SeMet, 33.9, DM	Dong et al., 2021
Apiaceae	Carrot	Selenite or selenate, ≤0.5 mM	Foliar spray	1.5 or 2.2, DM	SeMet, 0.43 or 0.37, DM	Kápolna et al., 2009
Brassicaceae	Broccoli, cauliflower, green cabbage, Chinese cabbage, kale, and Brussels sprouts	Selenate, 50 μM	Hydroponic application	160 on average, DM	SeMeCys, 80 on average, DM	Ávila et al., 2014
Brassicaceae	Broccoli	Selenate, 50 g ha^{-1}	Foliar spray	521–955, FM	SeMet and SeMeCys, 52–120, DM	Šindelářová et al., 2015
Brassicaceae	Broccoli	Selenate, 20 μM	Hydroponic application	801.2–1798.4, DM	SeMet and SeMeCys, about 0.5 $\mu M\,g^{-1}$ FM	Ramos et al., 2011
Brassicaceae	Cabbage	Selenate, 0.1 mM or 2.6 μM	Foliar spray or soil application	0.96 or 4.80, DM	SeMet, 0.18 or 2.52, DM	Mechora et al., 2012
Brassicaceae	Pak choi	Selenite, 10 μM	Hydroponic application	2.22, FM	SeMeCys, 0.61, FM	Yu et al., 2019
Brassicaceae	Pak choi	Selenate, 10 μM	Hydroponic application	42.17, FM	SeMet, 6.46 FM	Yu et al., 2019
Brassicaceae	Pak choi, kale, and broccoli sprouts	Selenate, <0.64 mM	Sand culture and nutrient supplement	155.9–467.1, DM	SeMeCys, 57.4–168.9, DM	Thosaikham et al., 2014
Brassicaceae	Radish	Nanoparticles, 12.7 μM	Hydroponic application	144, FM	SeMeCys, 43, FM	Palomo-Siguero et al., 2015
Brassicaceae	Radish	Selenate, ≤10 μM	Foliar spraying	120, DM	SeMeCys, 33, DM	Schiavon et al., 2016
Brassicaceae	White cabbage, broccoli, mustard, and rye sprouts	Selenium dioxide, 90.1 μM	Hydroponic application	53.3–400.0, DM	SeMet and SeMeCys, NG	Piekarska et al., 2014
Compositae	Lettuce	Selenite or selenate, ≤40 μM	Hydroponic application	50.8 or 602.0, DM	SeMet, 6.9 or 25.2, DM	do Nascimento da Silva et al., 2017
Leguminosae	Chickpea	Selenite or selenate, ≤40 g ha^{-1}	Soil application	0.70 or 2.92, DM	SeMet, 0.46 or 1.52, DM	Poblaciones et al., 2014
Leguminosae	Lentil and soy sprouts	Selenite and selenate (1:1), ≤23.1 μM	Hydroponic application	98–284, DM	SeMet, 14.9–29.1, DM	Funes-Collado et al., 2013
Leguminosae	Soybean	Selenite, 5 $\mu g\,g^{-1}$ soil	Soil application	75, DM	SeMet and SeCys, NG	Chan et al., 2010
Liliaceae	Garlic	Nanoparticles, 12.7 μM	Hydroponic application	About 22, DM	SeMeCys, About 16.06, DM	Li et al., 2020
Liliaceae	Garlic	NG	NG	1.36, DM	SeMeCys, NG	Kotrebai et al., 2000
Liliaceae	Onion	NG	NG	0.14, DM	SeMeCys, NG	Kotrebai et al., 2000
Liliaceae	Ramp	NG	NG	0.52, DM	SeMeCys, NG	Kotrebai et al., 2000
Solanaceae	Potato	Selenite or selenate, 100 g ha^{-1}	Foliar spray	0.78 or 1.22, DM	SeMet, 0.61 or 0.41, DM	Zhang et al., 2019b
Solanaceae	Potato	Selenate, 52.7 μM	Foliar spray	1.1, DM	SeMet, 0.33, DM	Cuderman et al., 2008

DM, dry matter; FM, fresh matter; and NG, not given.

while other vegetables tend to accumulate SeMet. For vegetables that mainly accumulate MeSeCys, the highest total Se concentration was found in broccoli ($1798.4 \pm 58.8\,\mu g\,g^{-1}$ DM) and the highest MeSeCys concentration was found in broccoli sprout ($168.9 \pm 19.0\,\mu g\,g^{-1}$ DM). For those mainly accumulate SeMet, the highest total Se concentration was found in lettuce ($602.0 \pm 6.0\,\mu g\,g^{-1}$ DM) and the highest SeMet concentration was found in Lion's Mane mushroom ($55.1 \pm 11.1\,\mu g\,g^{-1}$ DM). Studies reported that MeSeCys can be quickly converted into methyl selenol, which exerts putative anti-cancer activity (Ip et al., 2000). Therefore, vegetables from the *Brassicaceae* and *Liliaceae* families may represent suitable dietary sources for Se supplementation.

Percentage of Minimum Recommended Daily Allowance and Acceptable Daily Intake Based on Se Accumulation

Dietary intake of Se is largely dependent on the soil levels of Se, which vary in different regions. Daily Se intake is less than 11 μg in the Se-deficient regions of China where Keshan and

Kashin-Beck diseases occur (Liu et al., 2018); in contrast, the average Se intake is 550 μg per day in Enshi, China, a Se-rich region (Yuan et al., 2012; Huang et al., 2013). However, it was suggested that an intake of above 400 μg Se per day would lead to chronic toxicity (Winkel et al., 2012; Malagoli et al., 2015). Due to the narrow safe intake range, daily consumption of Se-enriched vegetables should be carefully considered. Therefore, based on the current biofortification methods (**Table 1**), we calculated the percentage of minimum recommended daily allowance and acceptable daily intake for Se-enriched vegetables and mushrooms (**Table 2**). The water contents of vegetables used in these calculations were taken from Food Data Central Database (United States Department of Agriculture Food Data Central Database, 2021). We found that 100 g of cabbage, carrot, chickpea, onion, or potato provide less Se than the minimum recommended daily allowance. The same quantities of other vegetables meet the minimum recommended daily allowance, and most exceed the acceptable daily intake. Therefore, it is important to control the Se content of vegetables within a suitable range and to increase the proportion of Se-AAs in vegetables.

FACTORS THAT AFFECT Se SPECIATION

Several authors reviewed factors that affect bioavailability of Se (the fraction of Se that is available for absorption by plant) in soil–plant system, including Se speciation, soil property (pH/Eh, metallic oxide, and organic matter and clay contents), plant condition (species, cultivar, and growth stage), climate condition, and agronomic management (tillage management, irrigation, rotation and intercrop management, and fertilizer). Such factors also have impacts on the Se speciation in plants. We summarized the related studies and discussed factors that affect Se speciation, including Se source, agronomic management, and vegetable species and cultivars.

TABLE 2 | Calculated percentage of minimum recommended daily allowance and acceptable daily intake for selenium-enriched vegetables and mushrooms.

Vegetable	Se content (μg 100 g^{-1} FM)	Percentage of MRDA (%)	Percentage of ADI (%)
Brassica sprouts	2026.7–6072.3	3684.9–11040.5	506.7–1518.1
Broccoli	8012.0–95,500	14567.3–173636.4	2003.0–4496.0
Cabbage	6.7–33.6	12.2–61.1	1.7–8.4
Carrot	14.9–21.8	27.0–39.6	3.7–5.4
Chickpea	2.8–11.7	5.1–21.2	0.7–2.9
Garlic	50.2–811.8	91.2–1476.0	12.5–203.0
Lettuce	269.2–3190.6	489.5–5801.1	67.3–797.7
Lentil and soy sprouts	3038.0–8804.0	5523.6–16007.3	759.5–2201.0
Mushroom	33.6–1118.0	61.1–2032.7	8.4–279.5
Onion	1.4	2.5	0.4
Pak choi	222.0–4217.0	403.6–7667.3	55.5–1054.3
Potato	17.2–26.8	31.2–48.8	4.3–6.7
Radish	480.0–14400.0	872.7–26181.8	120.0–3600.0
Soybean	405.0	736.4	101.3

MRDA, minimum recommended daily allowance and ADI, acceptable daily intake.

Se Source

Selenite, selenate, Se nanoparticles, and Se-AAs can be absorbed by vegetables (Dinh et al., 2019). They are assimilated into various Se metabolites in the cells of vegetables (**Figure 2**). Vegetables, such as turnip (Li et al., 2018a), lettuce (Hawrylak-Nowak, 2013), and green pea (Garousi et al., 2017), accumulate more Se contents by supplying selenate as compared with selenite. Studies also showed that selenate treatment resulted in more Se-AA contents in pak choi (Yu et al., 2019) and potato (Zhang et al., 2019b). Selenite and selenate are transported by phosphate transporters and sulfate transporters, respectively (Feist and Parker, 2001; Li et al., 2008; El Mehdawi et al., 2018), and compete with one another when they coexist. Selenite-inhibited selenate uptake and transport in wheat (Li et al., 2008), whereas high concentrations (0.63 μM) of selenate prevented selenite absorption in maize (Longchamp et al., 2013). The Se nanoparticles were found to be transported by aquaporins (Hu et al., 2018; Wang et al., 2020a), and their metabolic fate was similar to that of selenite (Palomo-Siguero et al., 2015; Hu et al., 2018; Wang et al., 2020a). It was suggested that Se-AAs may be transported by amino acid transporters (Kikkert and Berkelaar, 2013). However, vegetables contain dozens of amino acid transporters (Yang et al., 2020), and the specific amino acid transporters that transport Se-AAs have not yet been identified.

Inorganic Se as a Se Source for Biofortification

Selenite and selenate are frequently employed as inorganic sources of Se for plant biofortification. Absorbed selenite is rapidly converted into Se-AAs in the roots of wheat (Li et al., 2008) and pak choi (Yu et al., 2019), with only a small proportion of selenite being translocated to the shoots. In contrast, selenate is gradually converted into Se-AAs in the roots, with the majority of selenate being translocated to the shoots.

The Se nanoparticles have recently been proposed as a new type of fertilizer and can be synthesized *via* physical, chemical, or biological processes (Wadhwani et al., 2016; Skalickova et al., 2017) with varied particle sizes, stabilities, and bioavailabilities. Several studies have revealed that Se nanoparticles are oxidized to selenite in plants, implying that their metabolic fate is similar to that of selenite (Palomo-Siguero et al., 2015; Hu et al., 2018; Wang et al., 2020a). However, different plants exhibit distinct patterns of biotransformation of Se nanoparticles. Rice seedlings more efficiently biotransformed Se nanoparticles into Se-AAs than wheat seedlings which were grown in nutrient solutions in greenhouses (Hu et al., 2018; Wang et al., 2020a).

Even though inorganic sources of Se are largely used for vegetable biofortification, they have two disadvantages. Firstly, inorganic Se can only be used in low concentrations, otherwise stunting of root and plant growth, chlorosis, or withering may occur (Terry et al., 2000). Secondly, only some of the inorganic Se is biotransformed into Se-AAs. High proportions of the inorganic forms remain in vegetables (Thosaikham et al., 2014). Therefore, alternative strategies need to be explored to improve the bioavailability of inorganic Se to vegetables and to reduce the proportion of inorganic Se in the edible parts of vegetables.

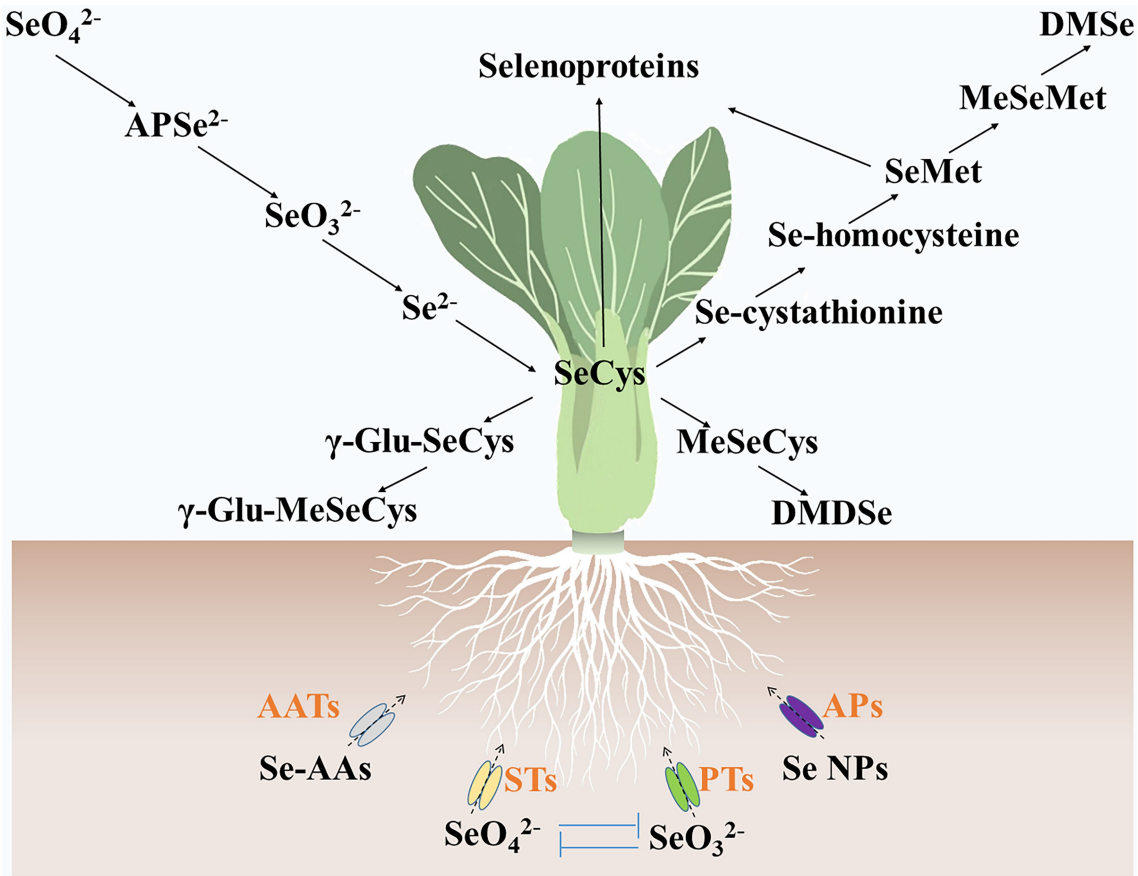

FIGURE 2 | Metabolic fate of selenium in vegetables. Se-AAs, selenate, selenite, and Se nanoparticles are absorbed by vegetables *via* amino acid transporters, sulfate transporters, phosphate transporters, and aquaporins, respectively. Then, these selenium forms were assimilated into various selenium metabolites, such as adenosine phosphoselenate, SeCys, γ-glutamyl-selenocysteine, γ-Glu-MeSeCys, MeSeCys, Se-cystathionine, Se-homocysteine, SeMet, Se-methylselenomethionine, dimethyldiselenide, dimethylselenide, and selenoproteins. AATs, amino acid transporters; APs, aquaporins; PTs, phosphate transporters; and STs, sulfate transporters. SeO_3^{2-}, Selenite; $APSe^{2-}$, adenosine phosphoselenate; SeO_4^{2-}, selenate; Se NPs, Se nanoparticles; γ-Glu-SeCys, γ-glutamyl-selenocysteine; MeSeMet, Se-methylselenomethionine; DMDSe, dimethyldiselenide; and DMSe, dimethylselenide.

Organic Se as a Se Source for Biofortification

Organic Se is the main form in agricultural soils under different cropping systems in Enshi, China, which accounts for 56–81% of the total Se (Qin et al., 2017). They have distinct availabilities to different plants. Kikkert and Berkelaar (2013) determined that SeMet and SeCys were more readily absorbed by durum wheat and spring canola than inorganic Se. It was also shown that SeMet was more effective than selenate for the production of Se-enriched garlic and Indian mustard (Ogra et al., 2017). On the contrary, inorganic Se was more bioavailable to oilseed rape than Se-AAs (Ebrahimi et al., 2015). In addition, Eich-Greatorex et al. (2007) found no differences in the uptake of organic Se (Se yeast and SeMet) and inorganic Se (selenate) fertilizers in wheat, barley, and oats. These results imply that Se-AAs can be utilized by plants; however, there is limited detailed information on the absorption, translocation, and accumulation of Se-AAs in individual vegetables.

We hypothesize that Se-AAs, particularly water-soluble MeSeCys and methylselenomethionine, can be absorbed directly by plants as several families of amino acids transporters have been identified, including the amino acid transporter family, amino acid-polyamine-choline, mitochondrial carrier family, preprotein and amino acid transporters, and the divalent anion: Na^+ symporter (Rentsch et al., 2007). In addition, methionine was previously reported to hinder SeMet uptake (Sandholm et al., 1973), implying that SeMet and methionine share the same transporters. Frommer et al. (1993) identified a broad specificity amino acid permease (pAAP1) in *Arabidopsis thaliana* that transported proline *via* a process in which methionine was a strong competitor. Therefore, pAAP1 may be capable of transporting SeMet.

The Se hyperaccumulators, such as *Astragalus* and *Stanleya pinnata* species, are not edible, at least not directly. These plants are rich in MeSeCys or SeMet and hence could be employed as organic Se sources for vegetable biofortification (Bañuelos et al., 2015, 2016; Wu et al., 2015). The use of seed meals derived from Se-enriched mustard and canola was shown to increase the Se content of strawberry fruits (Banuelos and Hanson, 2010). Amendment of the soil with Se-enriched *Stanleya pinnata* led to production of carrots and broccoli with SeMet as the predominant organic Se compound (Bañuelos et al., 2015, 2016).

Wang et al. (2018) revealed that amendment of Se-enriched wheat straw and pak choi increased the soil respiration rate and resulted in increased levels of soluble Se, exchangeable Se, and fulvic acid-bound Se, all of which contributed to higher Se bioavailability. Importantly, Se-enriched plants should be pre-incubated in soils to ensure the bioavailability of Se (Stavridou et al., 2011); otherwise, a reduced efficiency of Se uptake and fertilizer recovery would be observed (Ebrahimi et al., 2019). Overall, the Se present in Se-enriched plants is accessible to plants, and thus, Se hyperaccumulators, such as *Astragalus* species, that grow in Se-rich areas have the potential to be employed as natural and green sources of Se; a better understanding of the metabolic fate of MeSeCys and SeMet would help to rationally develop Se resources and produce Se-enriched vegetables with high-Se-AA contents.

Agronomic Management

Agronomic management has been widely used to regulate the quality of vegetables (Yang et al., 2021). Current studies indicate that the method and timing of application of Se fertilizers, mineral elements in the rhizosphere, and external conditions affect the levels of Se-AAs in various plant species, though only a few studies have investigated these factors in vegetables. However, these results may provide practical guidance for production of Se-enriched vegetables with high-Se-AA contents.

Application Method and Timing of Se Fertilizers

The Se fertilizers are generally applied *via* foliar and root application. Foliar application offers the advantages of enhanced utilization efficiency associated with prevention of environmental pollution (Niu et al., 2021). Application of Se at a critical growth stage is essential to ensure higher plant Se-AA contents. Studies have shown that the levels of organic Se were 2-fold higher in rice grains sprayed with 75 g ha^{-1} of selenate or selenite at the full heading stage than plants treated at the late tillering stage (Deng et al., 2017). In wheat, foliar spraying of 20 g ha^{-1} selenate at the pre-filing stage increased organic Se (mainly SeMet) by 5.34% compared to spraying at the pre-flowering stage (Wang et al., 2020b). In blueberry plants, foliar application of 200 g ha^{-1} selenate or selenite during the young fruiting stage resulted in 12.9–16.6% higher organic Se levels than treatment during the coloring stage (Li et al., 2018b).

Even though foliar application has advantages, Yin et al. (2019) found that root application of Se led to 91.2–97.1% higher Se-AA levels in rice than foliar application. However, root application generally adds more Se fertilizer to the soil and water, which leads to concerns related to possible long-term environmental impacts (Tan et al., 2016). Therefore, it is critical to optimize the application methods for different vegetables.

Mineral Elements in the Rhizosphere

Duncan et al. (2017) found that nitrogen fertilizers increased the total Se content and the proportion of undefined Se by 60–70% but decreased the major form of organic Se (SeMet) in wheat grains. Additionally, phosphorus and sulfur have been established to compete for selenite and selenate absorption, respectively (Feist and Parker, 2001; Li et al., 2008; El Mehdawi et al., 2018). Interestingly, phosphorus application at 160 μg g^{-1} decreased the total and organic Se contents in the grains of winter wheat, while the proportion of organic Se increased by 7.2–15.1% compared to no phosphorus application (Nie et al., 2020). Although sulfur deficiency enhanced the total Se content of wheat seedlings supplied with 10 μM selenate, the contents of MeSeCys in the shoots and roots decreased by 74.9 and 82.3%, respectively (Huang et al., 2017).

External Condition

External conditions, such as climate conditions and soil properties, have influences on the bioavailability of Se through affecting the absorption of Se by vegetables and Se fraction and speciation in the soil (Dinh et al., 2019). Renkema et al. (2012) found that enhanced transpiration at around 50% relative humidity increased Se translocation by up to 6-fold in durum wheat and spring canola. Soil pH governs Se speciation and changes the charges of bioavailable Se (Nakamaru and Altansuvd, 2014; Ponton et al., 2018). SeMet becomes negatively charged as the pH increases from 7 to 10; therefore, SeMet is more bioavailable at high pH (Ponton et al., 2018). Numerous studies have also been conducted to determine the influence of selenobacteria (Se biofortification by certain bacteria in the soil) on the accumulation of Se in plants. Several genera, including Acinetobacter, Bacillus, Enterobacter, Klebsiella, Paenibacillus, Pseudomonas, and Stenotrophomonas, were isolated and identified as Se-tolerant (Acuña et al., 2013; Durán et al., 2014). Among them, Acinetobacter E6.2 produced elevated levels of SeMet (10.0 μg g^{-1} DM) and MeSeCys (3.8 μg g^{-1} DM) without causing oxidative stress (Durán et al., 2015). In addition, co-inoculation of selenobacteria with an arbuscular mycorrhizal fungus further enhanced Se accumulation (Durán et al., 2013, 2016). However, organic matter existed in the soil or organic amendments reduced Se bioavailability to plants by immobilizing Se (Li et al., 2017).

Overall, agronomic management represents a practical method of obtaining Se-enriched vegetables with high-Se-AA contents. Thus, it is important to investigate the optimal application methods and timing of Se fertilization for specific vegetable crops. Phosphorus and sulfur are essential to obtain high contents of Se-AAs, while nitrogen and organic matter should be carefully controlled. Moreover, even though high pH, enhanced transpiration, and selenobacteria have been shown to be beneficial for Se accumulation, the Se speciation and their contents were not determined in the previous studies. Future studies should focus on the precise effects of external conditions on the Se-AAs accumulated by vegetable crops.

Vegetable Species and Cultivars

Uptake and accumulation of Se are distinct in different plant species. The Se hyperaccumulators generally accumulate 10- to 100-fold higher levels of Se than Se non-accumulators. These plants exhibit higher Se to sulfur ratios (Se preference), organic Se to inorganic Se ratios, shoot to root Se ratios, and source to sink Se ratios (Pilon-Smits, 2017). By taking advantage of elevated selenocysteine methyltransferase (SMT) levels, Se hyperaccumulators produce methylated forms of SeMet and SeCys,

rather than integrating these Se-AAs into proteins (Pilon-Smits, 2017). The Se hyperaccumulators are also capable of converting SeCys to elemental Se and alanine (Guignardi and Schiavon, 2017). In addition, they have higher contents of hormones (jasmonic acid, salicylic acid, and ethylene) and enhanced levels of stress-resistance genes, which contribute to Se assimilation and tolerance (Pilon-Smits, 2017). As a result, Se is less hazardous to Se hyperaccumulators, and Se hyperaccumulators generally have higher total Se and Se-AA contents than other plants (Finley, 2005; Wiesner-Reinhold et al., 2017). Similar to Se hyperaccumulators, vegetables from the *Brassicaceae* and *Liliaceae* families accumulate more total Se and higher MeSeCys contents (**Table 1**). Moreover, vegetables from the *Brassicaceae* family are tolerant to high concentrations of inorganic Se (0.64 mM of selenate by nutrient supplementation, **Table 1**).

Cultivar also has influence on uptake and accumulation of Se. It was reported that high-Se rice cultivars have been shown to alter the mass flow and activate Se by increasing the rhizospheric pH and secreting organic acids into rhizosphere, which result in improved Se bioavailability (Zhang et al., 2019c). In addition, total contents of SeCys, MeSeCys, and SeMet in broccoli heads were cultivar dependent, ranging from 0.20 to 0.66 $\mu g\,g^{-1}$ DM (Šindelářová et al., 2015).

METABOLISM OF Se-AAs

Regulation of Se-AAs in Vegetables

Several molecular studies have been conducted to reveal the mechanisms of detoxification in Se hyperaccumulators. The Se hyperaccumulators have been found to mitigate Se toxicity by converting SeCys to methylated and/or volatile forms (**Figure 3A**), decomposing SeCys (**Figure 3B**), and preventing SeCys misincorporation (**Figure 3C**). These findings provide important information for molecular breeding of Se-enriched vegetables with high-Se-AA contents.

The Se hyperaccumulators may exhibit increased expression of Se-assimilation genes (Lima et al., 2018). They also predominantly contain water-soluble and non-protein forms of organic Se compounds, such as MeSeCys (Pilon-Smits, 2017). Researchers have identified *SMT* genes that specifically methylate selenocysteine and homocysteine in vegetables, such as broccoli (Lyi et al., 2005) and Indian mustard (Chen et al., 2019). Overexpression of *SMT* increased the levels of MeSeCys by more than 95.6% (with selenite as the Se source) or 72.4% (with selenate as the Se source) in tomato fruits (Brummell et al., 2011). Apart from the expression levels of *SMT*, the enzyme activity of SMT is also important. Sors et al. (2009) reported that the SMT of a non-accumulator *Astragalus drummondii* lacked activity *in vitro,* while insertion of mutations increased its activity. The *WRKY47* gene and cytokinin have been associated with Se tolerance in *Arabidopsis thaliana*. The *wrky47* mutants of *A. thaliana* are sensitive to Se stress; these mutants exhibit decreased expression of *HMT1* and *HMT3,* which share significant primary sequence homology with *SMT* (Wu et al., 2020). The *tps22* mutants have decreased exogenous levels of cytokinins, which resulted in increased *SMT* expression (Jiang et al., 2018). Moreover, overexpression of the genes encoding cystathionine-γ–synthase (*CGS*) and COQ5 methyltransferase (*COQ5-2*) increased the production of volatile Se compounds, resulting in a lower Se content and decreased toxicity, in Indian mustard and broccoli, respectively (Huysen et al., 2003, 2004; Zhou et al., 2009).

Decomposing SeCys avoids misincorporation of SeCys into proteins. Overexpression of the genes encoding NifS-like protein (*CpNifS*) and selenocysteine lyase (*cpSL*) enhanced conversion of SeCys into alanine and elemental Se in *A. thaliana* and Indian mustard, respectively (Van Hoewyk et al., 2005; Bañuelos et al., 2007). Decreasing misincorporation of SeCys may reduce toxicity and increase the content of Se-AAs. A variant cysteinyl-tRNA synthetase (*CysRS*) that reduced the frequency of SeCys misincorporation was identified in Se hyperaccumulator *Astragalus bisulcatus* (Hoffman et al., 2019).

In the absence of the abilities described above, Se non-accumulators are sensitive to Se supplementation, thus absorb and accumulate less total Se, and have lower Se-AA contents. Therefore, further studies are needed to select and

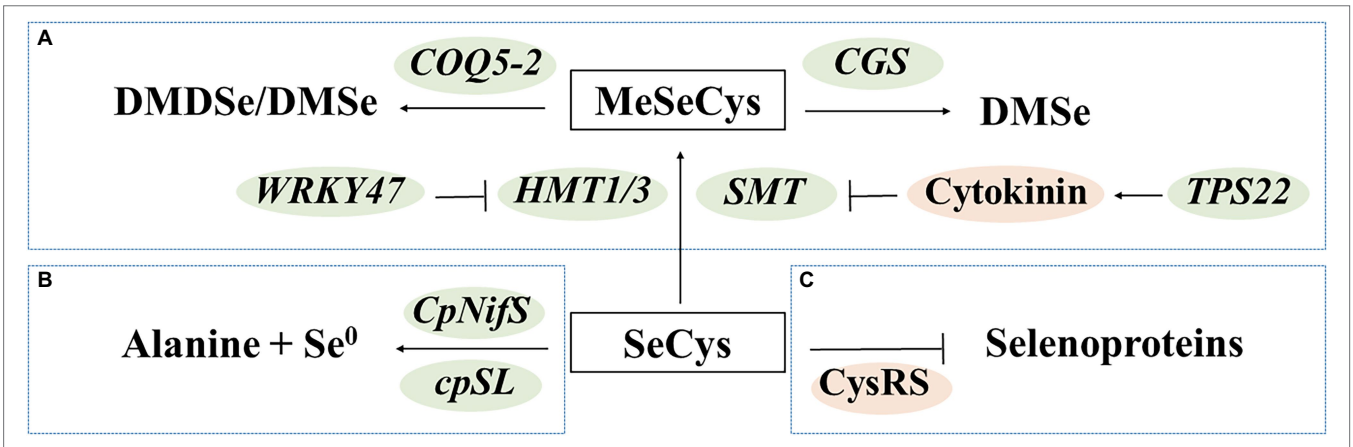

FIGURE 3 | Transgenic approaches to regulate seleno-amino acids in plants. **(A)** Production of methylated and/or volatile forms; **(B)** Decomposition of SeCys; and **(C)** Reduction of misincorporation. DMDSe, dimethyl diselenide; DMSe, dimethyl selenide; MeSeCys, Se-methylselenocysteine; and SeCys, selenocysteine.

breed vegetable cultivars with Se hyperaccumulator abilities. In addition, other genes involved in the transport and assimilation of inorganic Se have the potential to increase the content of Se-AAs. Researchers found that sulfate transporters (*Sultr*) and ATP sulphurylases (*APS*) play important roles in the accumulation of Se (Schiavon et al., 2015) and that overexpression of the phosphate transport gene (*OsPT8*) improved the Se content of *Nicotiana tabacum* (Song et al., 2017). However, it is a pity that the Se-AA contents were not determined in these studies.

Future Research Prospects

As described above, Se-AAs can be used as effective, green sources for Se biofortification. However, it is unknown whether Se-AAs are mineralized or directly absorbed by plants, what happens when Se-AAs are allocated into the roots and shoots, and how plants transport Se-AAs from their roots to the shoots (**Figure 4A**).

Researchers have generally addressed these issues by applying multiple analytical techniques (**Figure 4B**); however, these analytical techniques have some limitations. Isotope labeling has been used to investigate the metabolic fate and translocation of substances for many years, though the applicability of this approach is limited by the scarcity of isotopically labeled Se speciation (Pedrero and Madrid, 2009; Abdillah et al., 2021). The Se speciation is generally identified by coupling multiple techniques, such as high-performance liquid chromatography in conjunction with inductively coupled plasma mass spectrometry (HPLC-ICP-MS). Fluorescent probes have also been developed for the detection of Se-AAs (Abdillah et al., 2021). However, many probes perform poorly under the physiological conditions of living cells. Therefore, Guo et al. (2020b) designed an Au nanoparticle-based probe for detecting SeCys in plants, such as rice and tea.

The transporters of Se-AAs could be identified using high-throughput sequencing. Plant factories with artificial lighting (PFAL) system provide stable environmental conditions and can be used year-round to grow plants. Amino acid permeases that are responsible for the uptake and transport of Se-AAs could potentially be identified through precise management of environmental conditions and a variety of traits using PFALs. Overall, combined use of multiple technologies would be preferable for determining the mechanisms of uptake and translocation of Se-AAs in vegetables.

PERSPECTIVES

Improving the Se-AA contents of vegetables benefits human health and increases the economic value of vegetables. This review summarized previous research on the forms and metabolism of Se-AAs in vegetables. To produce safe and effective Se-enriched vegetables, a number of important issues still need to be elucidated, such as (1) how can we improve the accumulation of Se-AAs in vegetables and (2) how can we control the ratios of total Se and Se-AA contents of vegetables. Hereafter, we discuss these issues in detail and propose possible solutions.

Cultivar Selection as an Important Strategy to Improve Accumulation of Se-AAs

Biosynthesis of Se-AAs varies between different cultivars, including wheat (Duncan et al., 2017; Wang et al., 2021), potato (Cuderman et al., 2008), and pear (Bañuelos et al., 2012). It was reported that two rice cultivars supplied with SeMet accumulated significantly different Se contents (Zhang et al., 2006); the high-Se rice cultivar expresses more transporter genes and has a more optimal grain storage capacity than the low-Se rice cultivar (Zhang et al., 2020). In addition, accumulation of Se-AAs can be improved by enhancing their translocation. Amino acid permeases may function as

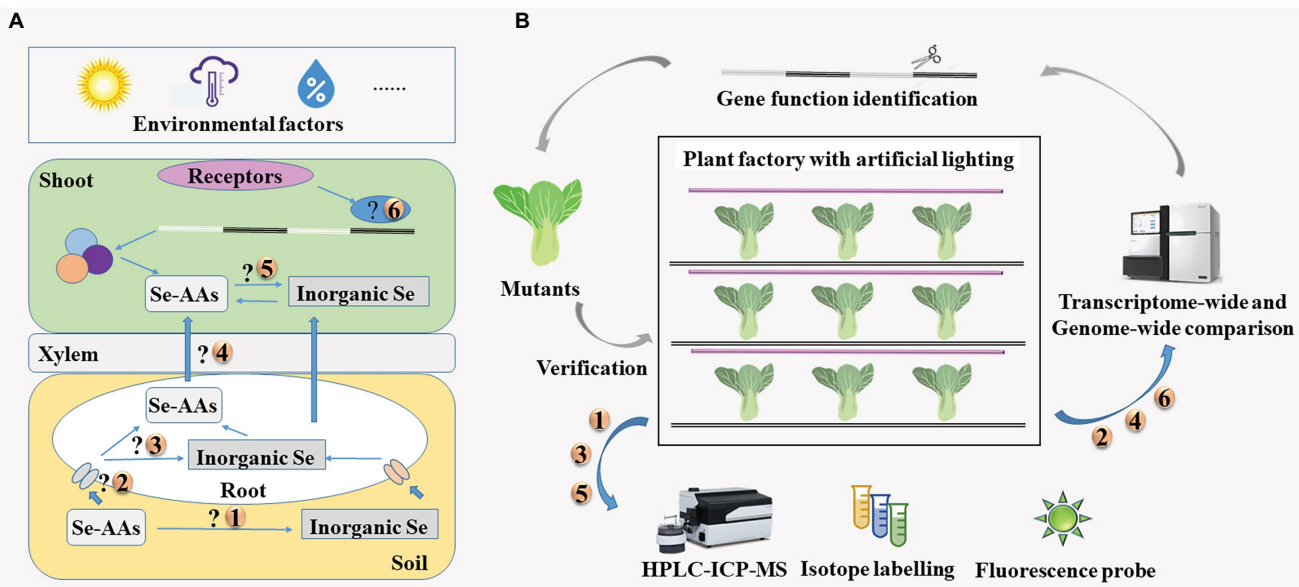

FIGURE 4 | (A) Key issues in research of seleno-amino acids in vegetables and **(B)** the proposed strategies to address these issues. HPLC-ICP-MS, high-performance liquid chromatography in conjunction with inductively coupled plasma mass spectrometry; Se-AAs, seleno-amino acids; and Se, selenium.

transporters of Se-AAs, as they transport amino acids from the soil into root cells and allocate the Se-AAs from the roots to shoots (Garneau et al., 2018; Guo et al., 2020a). As mentioned above, the majority of Se-AAs accumulated in the roots of wheat and pak choi. The Se-AAs did not appear to translocate to the shoots, which could be due to a lack of or low expression of amino acid permeases. These findings suggest that certain cultivars have a high capacity for Se-AA biosynthesis, translocation, and accumulation.

We propose that cultivar selection is an important strategy to obtain vegetable cultivars with a high capacity for Se-AA biosynthesis, translocation, and accumulation. However, conventional breeding strategies generally take a relatively long time. The PFALs could be considered as an ideal instrument to accelerate the breeding system. The PFALs allow the plant phenological period to be shortened by controlling important key factors, such as the lighting conditions, temperature, and nutrients supplied, to accelerate plant growth and development and to achieve the mature stage in a relatively short period. Indeed, five generations of short-day crops can be produced per year using PFALs (Jähne et al., 2020).

Regulation of Total Se and Se-AAs in Vegetables by PFAL and Artificial Intelligence

The PFALs equipped with intelligent decision support systems have the potential to be a valuable instrument for producing vegetables with the desired Se contents and high proportions of Se-AAs. Machine learning algorithms based on artificial intelligence have been widely used in agriculture, for applications, such as the prediction of gene function (Mahood et al., 2020), crop yield (Yoosefzadeh-Najafabadi et al., 2021), and heavy metal contents (Shi et al., 2016). However, there are no examples of machine learning to study the regulation of health-promoting components in vegetables.

Here, we propose a Precise Control of Se Content (PCSC) production system to regulate the total Se and Se-AA contents of vegetables (**Figure 5**). To initiate this system, the researchers

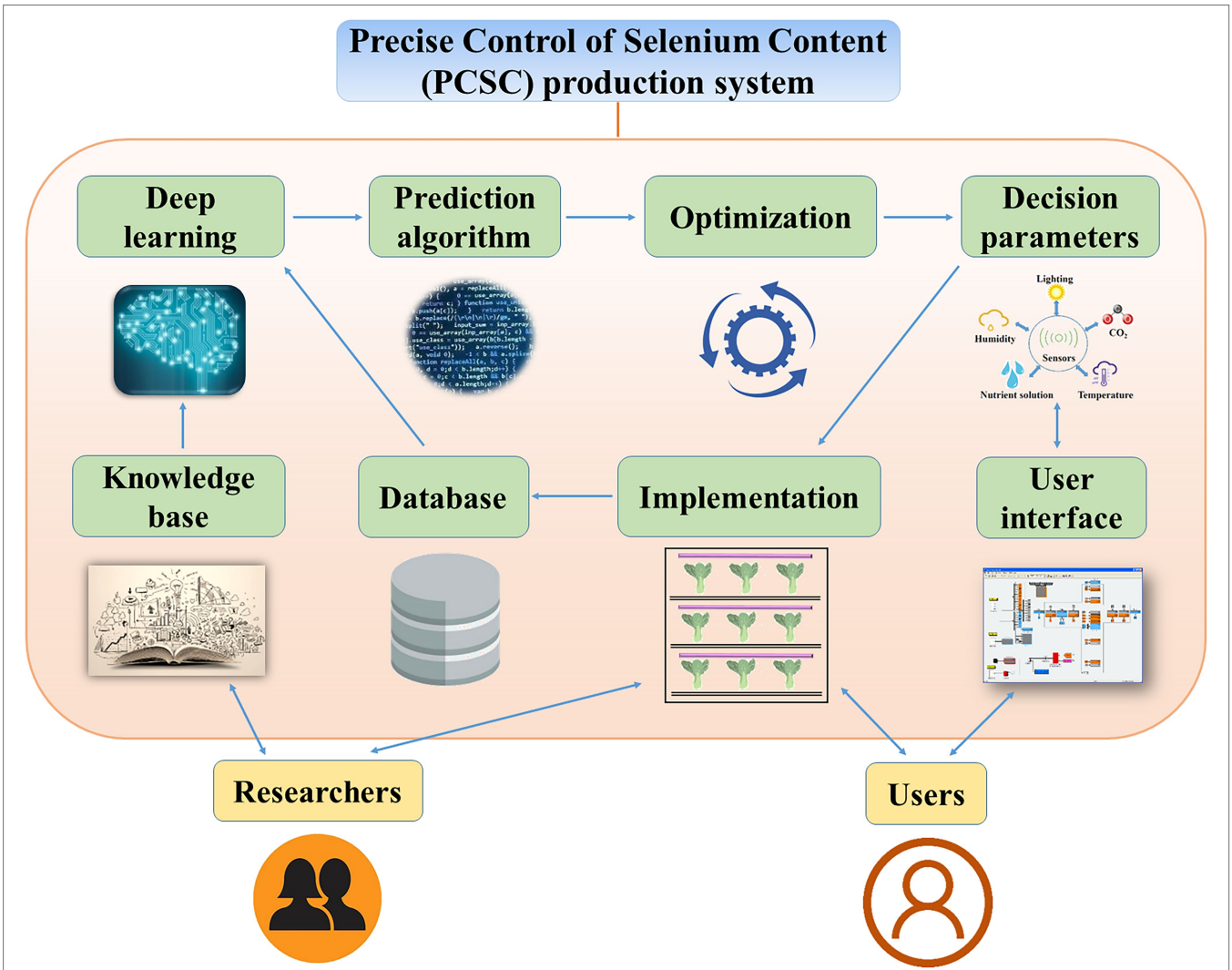

FIGURE 5 | A Precise Control of Selenium Content (PCSC) production system for precise control of the forms and selenium contents in vegetables.

would define the treatments based on the desired Se content for the vegetables. Experimental data on the forms of Se, lighting conditions, humidity, temperature, and cultivar would be collected to establish a knowledge base. Next, the knowledge base would be utilized in machine deep learning. The decision parameters could be obtained by selecting an appropriate prediction algorithm according to the users' objectives. However, to make more accurate decisions, an optimization model needs to be constructed. Then, the decision parameters would be tested in the PFAL. The results of the implementation would be collected in the database and would be subjected to further deep learning and prediction algorithm processes. After accumulation of vast data and repeated optimizations, the results would become increasingly accurate and therefore enable precise control of the total Se and Se-AA contents of vegetables. Although Se source, environmental condition, and plant species are known to affect the accumulation and regulation of the total Se and Se-AA contents of vegetables, detailed data and the precise mechanics remain vague. Therefore, further investigations are required to establish the proposed PCSC production system.

AUTHOR CONTRIBUTIONS

JH and XY created the hypothesis, objectives, outline the draft, and wrote the manuscript. ZW, LZ, JP, TH, BJ, and QY edited and added the discipline-specific feedback. All authors contributed to the article and approved the submitted version.

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Selenium Biofortification of Soybean Sprouts: Effects of Selenium Enrichment on Proteins, Protein Structure, and Functional Properties

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Selenium (Se) biofortification during germination is an efficient method for producing Se-enriched soybean sprouts; however, few studies have investigated Se distribution in different germinated soybean proteins and its effects on protein fractions. Herein, we examined Se distribution and speciation in the dominant proteins 7S and 11S of raw soybean (RS), germinated soybean (GS), and germinated soybean with Se biofortification (GS-Se). The effects of germination and Se treatment on protein structure, functional properties, and antioxidant capacity were also determined. The Se concentration in GS-Se was 79.8-fold higher than that in GS. Selenomethionine and methylselenocysteine were the dominant Se species in GS-Se, accounting for 41.5–80.5 and 19.5–21.2% of the total Se with different concentrations of Se treatment, respectively. Se treatment had no significant effects on amino acids but decreased methionine in 11S. In addition, the α-helix contents decreased as the Se concentration increased; the other structures showed no significant changes. The Se treatment also had no significant effects on the water and oil-holding capacities in protein but increased the foaming capacity and emulsion activity index (EAI) of 7S, but only the EAI of 11S. The Se treatment also significantly increased the antioxidant capacity in 7S but not in 11S. This study indicates that the dominant proteins 7S and 11S have different Se enrichment abilities, and the protein structures, functional properties, and antioxidant capacity of GS can be altered by Se biofortification.

Keywords: selenium biofortification, germinated soybean, protein structure, functional properties, antioxidant capacity

INTRODUCTION

Soybean is a major source of high-quality plant protein, providing essential amino acids and proteins with a variety of functional properties at low cost (1, 2). Soybeans also have multiple roles in food processing due to their unique protein-related food texture (3), high water-holding capacities, and foaming properties. Thus, the soybean has been widely used in the processing of sausages (4), beverages (5), bread, and cakes (6) to modify the food texture. The structure and functionality of soybean proteins are also important for the nutrition and quality of food products (7).

Selenium (Se) is an important micronutrient for both animal and human health, and Se intake should meet the recommended adult dietary allowance of 50–60 μg/day (8). However, the Se contents in agricultural soils are considerably low in some regions, such as China, New Zealand, and parts of Europe, where insufficient Se intake from plant-derived food has become a public health issue because of low Se levels in the environment (9). Selenoproteins play important roles in both human and animal health. For example, glutathione peroxidase (GPx) can protect the human body from oxidative stress (10). Indeed, the antioxidant activity of Se has attracted increasing research interest (11, 12), and the enrichment of foods through Se biofortification has become a popular strategy to promote adequate dietary intake of Se.

Excessive Se intake can result in Se toxicity, which largely depends on both the concentration of total Se and the Se speciation in dietary materials. Different Se species are associated with different metabolism pathways and further result in different levels of Se mobility, bioavailability, and toxicity (13, 14). In general, selenite (SeO_3^{2-}) is known to be more toxic to organisms than selenate and other organic Se compounds, such as selenomethionine (SeMet), selenocystine ($SeCys_2$), and methylselenocysteine (MeSeCys). Previous studies also found that these five Se compounds are the dominant chemical compounds of Se accumulated in Se-enriched soybean (7). Organic Se compounds have relatively low toxicity and high nutritional value compared with inorganic Se compounds (15), and those seleno-amino acids are incorporated into proteins, namely selenoproteins. Chan et al. found that over 80% of the total Se is bonded to high-molecular-weight proteins in Se-enriched soybean (16). Se-containing proteins in food products are ideal dietary sources of Se intake and are used as food supplement products *via* appropriate processing procedures in the agri-food industry (17).

However, Se is distributed unequally in protein fractions. Soybean proteins mostly consist of globulins, and four kinds, namely, 2S, 7S, 11S, and 15S, account for 15, 34, 41.9, and 9.1% of all soybean globulins, respectively. 7S and 11S account for 75.9% of the total globulins (18), as the dominant proteins, they have been the focus in regards to soybean and protein processing in previous studies (19, 20). A previous study found that the abilities of different protein fractions to enrich Se differ from each other: the concentration of Se in 11S was 38% higher than that in 7S in soybeans cultivated by Se foliar spray, and 11S had a higher Se enrichment ability than 7S (7). However, there is less information on the distribution and species of Se in Se biofortification-germinated soybean proteins, and the Se distribution and these species should be clarified.

To better utilize Se-enriched germinated soybean protein, the structure of the protein should be known. Previous studies have indicated that protein structure, functional properties, and antioxidant capacity are changed during germination (21–23). Simultaneously, Se can bind to soybean proteins by S–S, Se–S, and Se–Se bonds; therefore, parts of the secondary structure, such as the α-helix, β-sheet, and random coil, are influenced (24). A previous study found that Se could influence the secondary structures of 7S and 11S in raw soybeans (25), but Deng et al. found that there was no significant effect on protein secondary structure in soybeans enriched through Se foliar spraying (7). The influence of Se on the secondary structure of raw soybean protein has not yet been clarified, and to the best of our knowledge, Se-enriched germinated soybean proteins have not been studied.

Changes in protein components and structure can affect their functional properties and antioxidant capacity. The functionality of soybean proteins, including their water-holding capacity (WHC), oil-holding capacity (OHC), foaming capacity (FC), and emulsion activity index (EAI), will influence how soybean proteins work in food processing. Germination can cause the breakdown of macro-molecular proteins, and the secondary structure of the protein changes after germination, affecting both the FC and EAI of proteins (26). Previous studies have focused on the functionality of Se-enriched soybean (7, 25), but less is known about germinated Se biofortification soybeans. Previous studies investigated the antioxidant capacity of germinated soybean (22, 27) and found that germination could improve the antioxidant capacity. The antioxidant capacity of Se-containing protein from *Ganoderma lucidum* was three times higher than that of the proteins without Se treatment. In addition, the capacity was correlated with the Se content in protein (28). However, the effect of Se biofortification on soybean sprout proteins remains unknown.

As the application of Se biofortification during germination in the food industry has increased, Se distribution and its effects on protein structure and functionality need to be clarified. Previous studies have confirmed that Se biofortification can promote the content of Se and influence the protein structure and functional ability of Se-enriched soybeans; however, its effects on germinated soybean proteins remain unclear. In addition, few studies have assessed Se distribution and speciation in biofortified germinated soybean proteins and their effects on the structure of different soybean protein fractions. Therefore, the purpose of our study was to investigate the effects of Se on the structure, functional properties, and antioxidant capacity of germinated soybean proteins.

MATERIALS AND METHODS

Selenium-Enriched Soybean Sprouts

Soybean (Zhonghuang 13) seeds were provided by the Institute of Crop Science of CAAS (Beijing, China). The soybean seeds were surface-sterilized with 0.1% NaClO solution for 5 min, then washed five times with deionised water (Milli-RO Plus; MilliporeSigma, Burlington, MA, USA), and soaked in different concentrations of Se solution (0, 5, 30, and 60 mg/L of sodium selenite solution) at a ratio of 1:5 (w/v) for 6 h. Later, the seeds were placed in plant tissue culture containers with two layers of gauze and then placed in an incubator in the dark with a controlled temperature of 25°C. The seeds were sprayed with deionised water (∼350 ml) for 5 s every 10 h during germination, and soybean sprouts were harvested every 24 h until 120 h. Raw soybean (RS), germinated soybean in the control (GS), and germinated soybean under Se treatment (GS-Se5/30/60) were freeze-dried and ground with a grinder to pass through a 40 mesh sieve. The powders were sealed in bags until use. 7S and 11S were

prepared according to the previous methods (7, 29), which are presented in the **Supplementary Material**.

Total and Species Se Analysis

Approximately 0.5 g of soybean sprout powder was mixed with 6 ml of HNO_3 and 2 ml of H_2O_2 (30%) in 50-ml polypropylene tubes. The mixture was digested at 120°C until white fumes appeared. Concentrated HCl (5 ml) was added as a reductant to reduce selenate to selenite. The solution was diluted with deionised water to 25 ml, and Se was detected using hydride generation atomic fluorescence spectrometry (HG-AFS 9230; Beijing Titan Instruments, Beijing, China). The limits of quantification and detection for Se based on plant dry weight for the entire procedure were estimated to be 0.48 and 0.36 μg/kg, respectively.

Samples were prepared according to a previous study (30), with some modifications. A powdered sample (0.1 g) was transferred into a 15-ml plastic tube, and 10 ml of Tris-HCl (75 mmol/L, pH 7.5) and 10 mg protease XIV (Sigma-Aldrich, St. Louis, MO, USA) were added. The mixture was homogenized using ultrasound at 37°C for 18 h. The supernatant was collected after being centrifuged, and the filtrate was obtained by a 0.22-μm hydrophilic filter and stored at 4°C until Se speciation analysis.

Instrumental analysis was conducted according to a previous study (31), with some modifications. The Se species were determined using a high-performance liquid chromatography (HPLC) system (U3000; Thermo Fisher Scientific, Waltham, MA, USA) equipped with a ZORBAX SB-Aq column (4.6 × 250 mm, particle size, 5 μm; Thermo Fisher Scientific). The flow rate of mobile phase (10 mM citric acid, 0.5 mM sodium 1-hexanesulfonate, 2% methanol, pH 5.5) was 0.8 ml/min. The outlet of the HPLC system was coupled to an ICP-MS instrument (X Series 2; Thermo Fisher Scientific; USA). The column outlet of the HPLC system was connected to a Micro-mist nebuliser using PEEK tubing (0.25 mm i.d. × 10^4 cm length).

Amino Acids and Protein Subunit Analysis

Amino acids in samples were determined as previously reported (7) and are presented in the **Supplementary Material**. The protein subunit was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to a previous study (26), with minor modifications. Then, 2 mg/ml soybean protein sample solution was first prepared, and the buffer was 12% separating gel and stacking gel. After being stained, gels were decolorised until the background was clear. The standard marker was a 10–1,000 kDa molecular weight protein.

Fourier Transform Infrared Spectroscopy Analysis

Fourier transform infrared (FTIR) spectroscopy analysis of protein was performed according to a previous study (30). Approximately 1 mg dried sample was mixed with 100 mg KBr and pressed into a pellet. The pellet was analyzed by a Nicolet 5700 FTIR spectrometer (400–4,000 cm^{-1} wavenumber) with a 4 cm^{-1} resolution and an accumulation of 32 scans. Data were acquired and processed using Omnic 8.0 software (Thermo Fisher Scientific Inc., Madison, WI, USA) and Peakfit 4.12 (Systat Software, San Jose, CA, USA).

Water and Oil-Holding Capacity Analysis

The WHC and OHC were determined according to a previous report (7). About 0.5 g of sample (W_0) was placed into a centrifuge tube (25 ml) and then weighed (W_1). Then, 10 ml water or oil was added, and the sample was allowed to sit for 30 min before centrifuging for 10 min (6,000 × *g*). The samples together with the centrifuge tube were weighed after removing the upper layer of water or oil (W_2). WHC and OHC were calculated using the following equation:

$$WHC\,(OHC) = \frac{W_2 - W_1}{W_0} \quad (1)$$

Foaming Capacity and Foam Stability Analysis

FC and FS were assessed according to a previous report (32). A 30 ml sample of 1% (w/v) protein solution (pH 7.0) was homogenized in a mechanical homogeniser at 13,000 rpm for 3 min. FC was calculated using the following equation:

$$FC\,(\%) = \frac{V_1 - V_0}{V_0} \quad (2)$$

$$FS\,(\%) = \frac{V_2 - V_0}{V_1} \quad (3)$$

where V_0 and V_1 are the volumes before and after whipping, respectively, and V_2 is the volume after standing for 30 min.

Emulsifying Activity Index and Emulsifying Stability Index Analysis

EAI and ESI were assessed according to a previous report (33) and calculated using Equations 4, 5. Aqueous emulsifier solution (9 ml, containing 1% protein) and 3 ml soybean oil were added into a tube and blended. Then, 20 μl of the emulsion was pipetted into 5 ml of 0.1% sodium dodecyl sulfate aqueous solution. Then, the absorbance was read at 500 nm at 0 (A_0) and 30 (A_{30}) min.

$$\mathbf{EAI}\,\left(\mathbf{m2/g}\right) = \frac{\mathbf{2(T \times A_0 \times N)}}{\mathbf{C \times \Phi \times 10000}} \quad (4)$$

$$\mathbf{ESI}\,(\mathbf{min}) = \frac{\mathbf{A_0 \times t}}{\mathbf{A_0 - A_{30}}} \quad (5)$$

where T is 2.303, A_0 and A_{30} are the absorbances at 0 and 30 min, respectively, N is 250, C is the initial protein concentration, and Φ is the volume fraction of the emulsion (0.2).

Antioxidant Activity Analysis

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability of 7S and 11S were analyzed according to a previous study (34). Briefly, 2.0 ml of a water solution of the samples at 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml was mixed with 2 ml alcoholic solution of DPPH (1.0 × 10^{-4} M) and then measured after the reaction.

The DPPH radical-scavenging ability was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_2 - A_1}{A_0}\right] \times 100\% \quad (6)$$

where A_0 is the absorbance of the deionised water; A_1 and A_2 are the absorbances of the sample with ethanol solution and DPPH, respectively.

The •OH radical-scavenging ability of 7S and 11S was analyzed according to a previous study (35). Briefly, 2.0 ml of a water solution of the samples at 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml were mixed with 1.0 ml of 9.0 mmol/L salicylic acid ethanol solution and 1.0 ml of 9.0 mmol/L $FeSO_4$ and H_2O_2 aqueous solution and then measured after the reaction. The •OH radical-scavenging ability was calculated by the following equation:

$$\text{OH radical scavenging ability (\%)} = 1 - \frac{A_2 - A_1}{A_0} \times 100\% \quad (7)$$

where A_0 is the absorbance of deionised water, and A_1 and A_2 are the absorbances of the sample with deionised water and H_2O_2, respectively.

Data Analysis

Analysis of variance was performed followed by Duncan's test ($p < 0.05$) in the SPSS 19 software (IBM Corp., Armonk, NY, USA). For each tested group, the sample sizes in "amino acid concentration" are 2, and the other sample sizes are 3, respectively. Figures were drawn by Origin 2018 (OriginLab Inc., Northampton, MA, USA).

RESULTS

Total Se in Soybean and Its Proteins

The total Se of soybean/sprout powder (SP), 7S, and 11S in RS, GS, and GS-Se with different concentrations of Se treatment is summarized in **Table 1**. There was an increase in total Se content, as the Se concentration in the solution increased from 5 to 60 mg/L; the total Se in SP, 7S, and 11S of GS-Se60 was 2,882, 2,035, and 4,301 μg/kg, and elevated by 79.8, 60.2, and 73.9 times, respectively, compared with the control group. The total Se in 11S was significantly higher than that in 7S by 69.6, 72.1, and 111% in RS, GS, and GS-Se30, respectively.

Se Species in Different Proteins

Five Se species were separated and identified by HPLC-inductively coupled plasma mass spectrometry; the chromatograms are shown in **Figure 1** (A, standard solution; B, sample). The retention times of standard $SeCys_2$, MeSeCys, SeMet, selenite, and selenate were 2.694, 3.146, 3.832, 4.937, and 13.019 min, respectively, and the recoveries were calculated through the sum of different Se species to total Se ranging from 77.7 to 97.5%. Of the five Se species, SeMet and MeSeCys were the dominant Se species (**Figure 2**), accounting for 41.5–80.5 and 19.5–21.2% in GS-Se with different concentrations of Se treatment, respectively. However, $SeCys_2$ was only found in the germinated soybean with Se treatment above 30 mg/L, and selenate was not found in any of the treatments. The proportion of organic Se (sum of $SeCys_2$, MeSeCys, and SeMet) decreased from 100 to 67.8%, as the Se treatment increased from 0 to 60 mg/L, and the proportion in 7S and 11S was higher than that in SP; however, the percentage of organic Se in 7S and 11S was 76.2 ± 4.9% and 77.7 ± 6.0%, respectively. There was no significant difference in the proportion of organic Se between the two protein fractions at the same level of Se treatment. Furthermore, the concentrations of different species were all higher in 11S than those in 7S by 31.5–150%.

TABLE 1 | Total selenium (Se) concentration in raw soybean (RS), germinated soybean (GS), and germinated soybean with Se biofortification (GS-Se) with different Se treatment concentrations (μg/kg).

Samples	Soybean/sprouts	7S	11S
RS	33.03 ± 2.44 b	30.41 ± 1.26 b	51.59 ± 2.23 a
GS	36.12 ± 2.57 b	33.82 ± 0.98 b	58.21 ± 4.40 a
GS-Se5	242.5 ± 12.74 b	227.3 ± 14.66 b	357.0 ± 22.10 a
GS-Se30	1,297 ± 35.53 b	952.1 ± 37.34 c	2,040 ± 40.98 a
GS-Se60	2,882 ± 46.01 b	2,035 ± 90.51 c	4,301 ± 214.5 a

Different letters are used to show significant differences at various treatments ($p < 0.05$).

Changes in Amino Acid Content

The amino acid composition of soybean sprouts subjected to Se biofortification is listed in **Table 2**. The content of glutamic acid was the highest in all treatment groups (17.42–23.70%), followed by aspartic acid (10.71–12.41%), while the contents of cysteine (0.82–1.09%) and methionine were the lowest (0.72–1.45%). Isoleucine and leucine showed an increasing trend after germination, while most of the other amino acids increased slightly, without significant differences. The total amino acid content increased significantly after germination in both 7S and 11S. Se treatment had no significant effects on amino acids but decreased methionine in 11S. The amino acid content in 7S was significantly higher than that in 11S.

Subunit Composition of Proteins

The subunits of different protein fractions (7S and 11S) from RS, GS, and GS-Se60 were analyzed by SDS-PAGE, and the results are shown in **Figure 3**. The molecular weight of 7S was mainly distributed at ∼70, 40, 35, and 15 kDa, and the molecular weight of 11S was mainly distributed at ∼37, 34, and 17 kDa. Both 7S and 11S shared a similar subunit composition in RS, GS, and GS-Se60, and there was no disappearance of the protein bands or appearance of new protein bands under Se treatment. Moreover, 7S and 11S were partially degraded into peptides with a low molecular weight due to germination, and the molecular weights of some proteins were lower than 15–25 kDa.

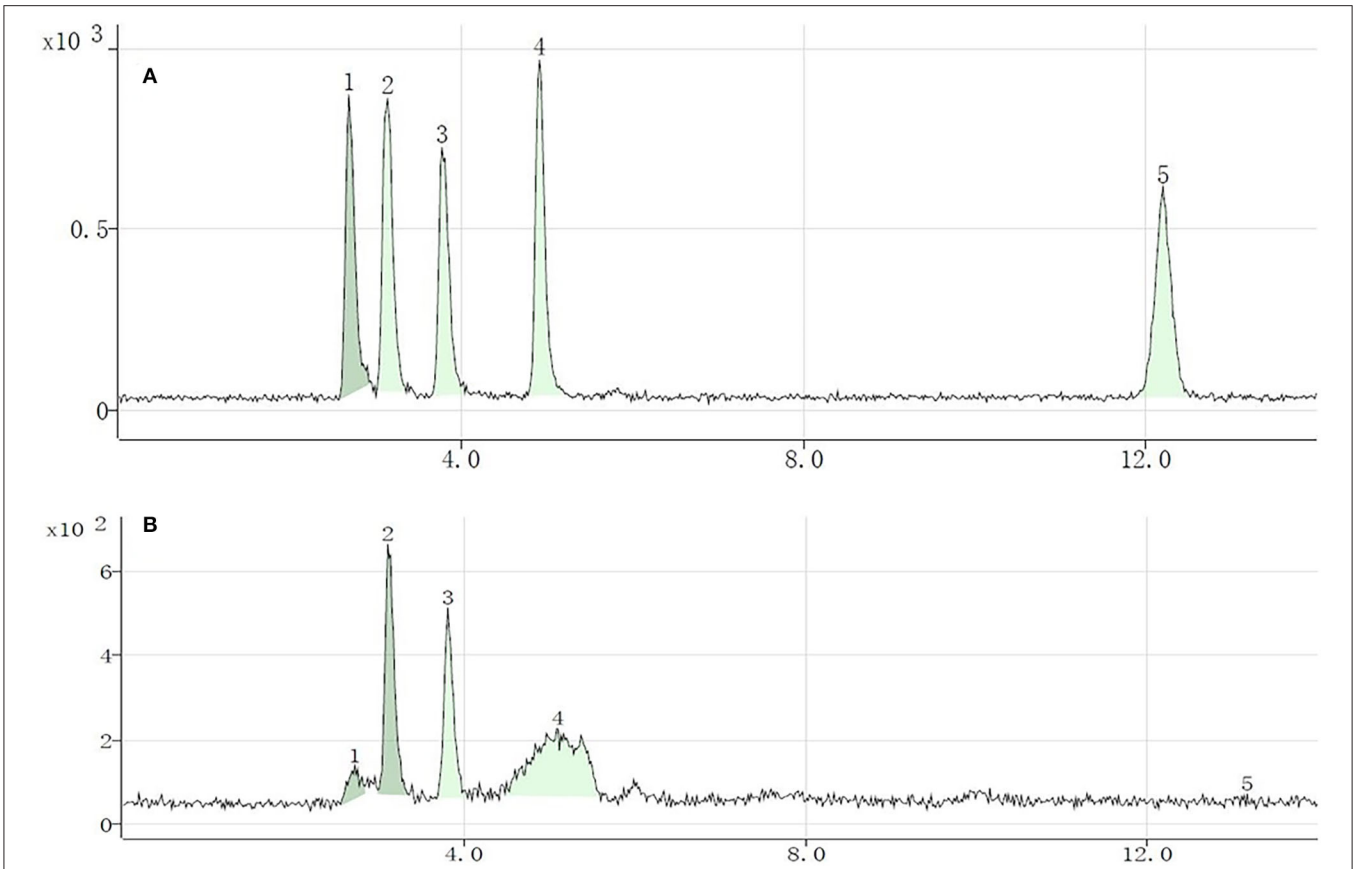

FIGURE 1 | Chromatograms of different selenium (Se) species in standard solution (10 μg/L) **(A)** and Se-enriched germinated soybean sprouts **(B)**. Selenocystine (1), methylselenocysteine (2), selenomethionine (3), selenite (4), and selenate (5).

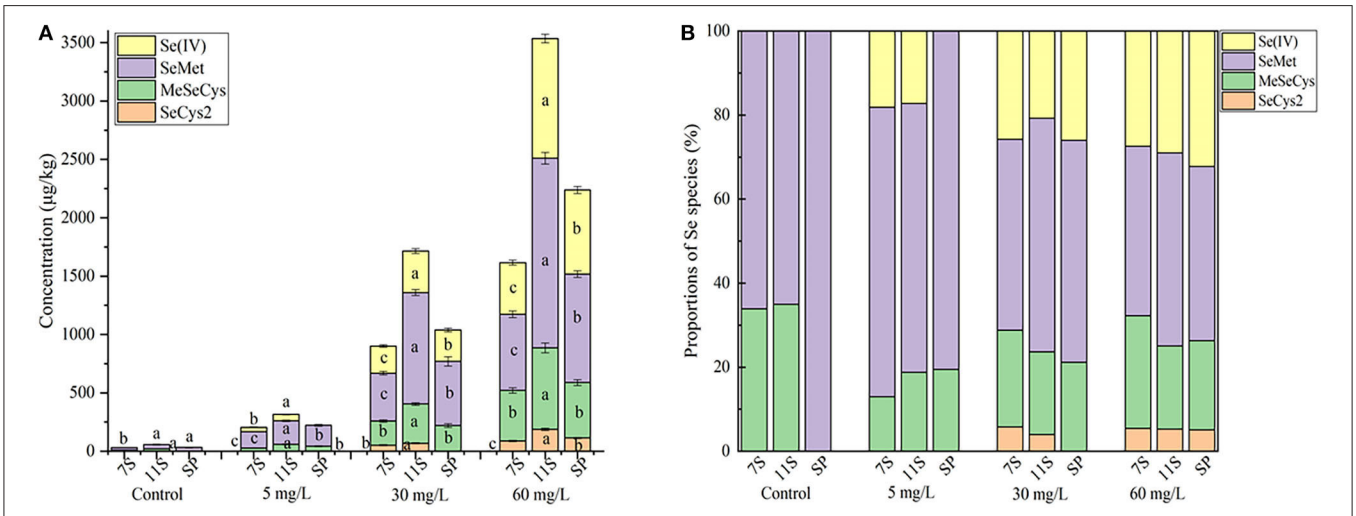

FIGURE 2 | Concentration **(A)** and proportions **(B)** of Se species in soybean and proteins under different treatments. Different letters are used to show significant differences at various treatments ($p < 0.05$).

Secondary Structural Composition of Different Soybean Proteins

The percentages of α-helix, β-sheet, β-turn, and random coil secondary structures of different protein fractions determined by FTIR spectroscopy in all the samples are shown in **Table 3**. The order of the secondary structure of proteins in all treatments was as follows: β-sheet, β-turn, random coil, and α-helix. Compared to those in RS, germination increased secondary structural

TABLE 2 | Total amino acid concentration during soybean germination with different concentrations of Se treatment (g/100 g).

Amino acids	7S-RS	7S-GS	7S-GS-Se30	11S-RS	11S-GS	11S-GS-Se30
Aspartic (Asp)	9.01 ± 0.35 a	9.70 ± 0.41 a	9.23 ± 0.16 a	6.23 ± 0.18 a	6.52 ± 0.42 a	6.66 ± 0.28 a
Threonine (Thr)	2.46 ± 0.20 b	2.50 ± 0.16 b	2.52 ± 0.10 b	2.92 ± 0.14 a	2.95 ± 0.06 a	2.82 ± 0.20 ab
Serine (Ser)	3.36 ± 0.16 a	3.37 ± 0.08 a	3.34 ± 0.14 a	2.55 ± 0.04 b	2.44 ± 0.16 b	2.38 ± 0.07 b
Glutamate (Glu)	17.88 ± 0.83 a	17.99 ± 0.54 a	17.05 ± 0.34 a	11.90 ± 0.25 b	11.55 ± 0.33 bc	10.50 ± 0.31 c
Proline (Pro)	3.00 ± 0.10 a	3.05 ± 0.18 a	3.04 ± 0.16 a	1.91 ± 0.03 b	2.07 ± 0.17 b	2.21 ± 0.10 b
Glycine (Gly)	3.56 ± 0.13 c	3.56 ± 0.17 c	3.66 ± 0.14 bc	4.08 ± 0.13 a	4.01 ± 0.11 a	3.92 ± 0.08 ab
Alanine (Ala)	2.95 ± 0.06 a	2.97 ± 0.16 a	2.88 ± 0.20 a	2.72 ± 0.08 a	2.78 ± 0.07 a	2.89 ± 0.13 a
Cysteine (Cys)	0.65 ± 0.06 a	0.64 ± 0.06 a	0.55 ± 0.03 a	0.62 ± 0.01 a	0.65 ± 0.04 a	0.63 ± 0.03 a
Valine (Val)	2.98 ± 0.10 ab	3.14 ± 0.06 a	2.85 ± 0.06 b	2.90 ± 0.10 b	2.89 ± 0.01 b	2.45 ± 0.13 c
Methionine (Met)	0.54 ± 0.03 d	0.75 ± 0.04 bc	0.71 ± 0.01 bc	0.79 ± 0.04 b	0.88 ± 0.04 a	0.67 ± 0.03 c
Isoleucine (Ile)	2.51 ± 0.10 b	2.92 ± 0.11 a	2.67 ± 0.10 b	1.91 ± 0.10 d	2.27 ± 0.03 c	2.02 ± 0.11 d
Leucine (Leu)	7.00 ± 0.25 b	7.79 ± 0.25 a	7.79 ± 0.08 a	5.02 ± 0.18 d	6.03 ± 0.21 c	6.24 ± 0.17 c
Tyrosine (Tyr)	2.89 ± 0.24 ab	3.07 ± 0.17 a	3.00 ± 0.10 ab	2.48 ± 0.04 c	2.71 ± 0.13 abc	2.63 ± 0.14 bc
Phenylalanine (Phe)	3.32 ± 0.08 a	3.31 ± 0.13 a	3.12 ± 0.13 a	2.35 ± 0.18 b	2.48 ± 0.04 b	2.25 ± 0.11 b
Lysine (Lys)	4.11 ± 0.14 a	4.20 ± 0.11 a	4.02 ± 0.18 a	2.60 ± 0.16 b	2.77 ± 0.07 b	2.55 ± 0.11 b
Histidine (His)	2.64 ± 0.10 c	2.74 ± 0.16 c	2.55 ± 0.16 c	1.68 ± 0.14 d	4.02 ± 0.13 b	6.02 ± 0.21 a
Arginine (Arg)	6.58 ± 0.10 a	6.47 ± 0.31 a	5.98 ± 0.13 b	4.11 ± 0.11 c	3.83 ± 0.16 cd	3.42 ± 0.11 d
Total	75.44 ± 0.03 b	78.17 ± 1.20 a	74.96 ± 0.34 b	56.77 ± 1.24 d	60.85 ± 1.44 c	60.26 ± 0.47 c

Different letters are used to show significant differences at various treatments ($p < 0.05$).

components of different proteins in α-helix and β-turn and decreased them in β-sheet and random coil in 7S. Conversely, germination increased secondary structural components of proteins in random coil and decreased them in α-helix, β-sheet, and β-turn in 11S compared with those in RS. Comparing the structural composition percentages in the control and Se biofortification samples, the α-helix decreased with an increase in the Se concentration, while the other structural compositions were not significantly different, and the secondary structures of 11S showed no significant differences between the control and Se biofortification samples.

Functionality of Different Protein Fractions

The WHC and OHC of proteins from GS were significantly higher than those from RS, increasing by 18.17–20.28% in WHC and 10.40–27.32% in OHC, while Se treatment showed no significant effects on either WHC or OHC within the same treatment. The WHC of 7S and 11S from GS-Se60 was not significantly different; however, the OHC of 7S was significantly higher than that of 11S, increasing by 23.28–42.17% (**Figures 4A,B**). The FC of 7S increased with germination, and Se treatment promoted the FC of 7S (**Figure 4C**); however, there were no significant differences in 11S. Germination showed no significant effects on FS in both 7S and 11S (**Figure 4D**); the FS in 7S increased under Se treatment but showed no significant differences in 11S. The EAI of 7S and 11S decreased after germination, whereas Se biofortification had no significant effects on either 7S or 11S (**Figure 4E**). The ESI increased significantly under Se biofortification, whereas Se had no significant effects on 11S (**Figure 4F**).

Antioxidant Activities

Germination promoted the ability of soybean protein to scavenge DPPH free radicals and hydroxyl radicals in both 7S and 11S, and the DPPH and hydroxyl radical-scavenging activities of the samples increased in a concentration-dependent manner (**Figure 5**). Se improved the ability of soybean protein to scavenge DPPH free radicals and hydroxyl radicals in 7S; however, this ability decreased in 11S. The order of DPPH scavenging ability among different proteins at 5 mg/ml was as follows: 11S-GS (51.8 ± 1.69), 7S-GS-Se60 (48.1 ± 0.99), 11S- GS-Se60 (40.1 ± 0.94), 7S-GS (37.0 ± 0.49), 11S-RS (28.3 ± 2.16), and 7S-RS (14.5 ± 0.85). The order of hydroxyl radical-scavenging ability among different proteins at 5 mg/ml was as follows: 11S-GS (35.74 ± 1.81), 7S-GS-Se60 (34.1 ± 1.40), 11S-GS-Se60 (34.08 ± 1.40), 11S-RS (33.18 ± 1.13), 7S-GS (30.1 ± 1.40), and 7S-RS (22.5 ± 1.13).

DISCUSSION

Total and Species Se Distribution in Different Proteins

The results indicated that germination in the presence of a selenite solution is an efficient method for Se biofortification of germinated soybeans, similar to a previous study (36) in which the Se concentration in soybean increased from 4.6 to 10,100 μg/kg. Se is beneficial at low levels but toxic at high levels, and the margin between deficiency and excess is narrow (13). To achieve health benefits, it has been suggested that Se intake should exceed the adult recommended dietary allowance of 50–60 μg/day and stay below the tolerable upper intake level of 400 μg/day (37). In our study, the concentration of total Se in GS-Se60 was 2,822 μg/kg, which was 79.8 times higher than that in RS. The ability of 11S to enrich Se was markedly higher than that of 7S in all treatment groups during germination. To the best of our knowledge, there has been no study investigating Se distribution in different germinated soybean proteins, and

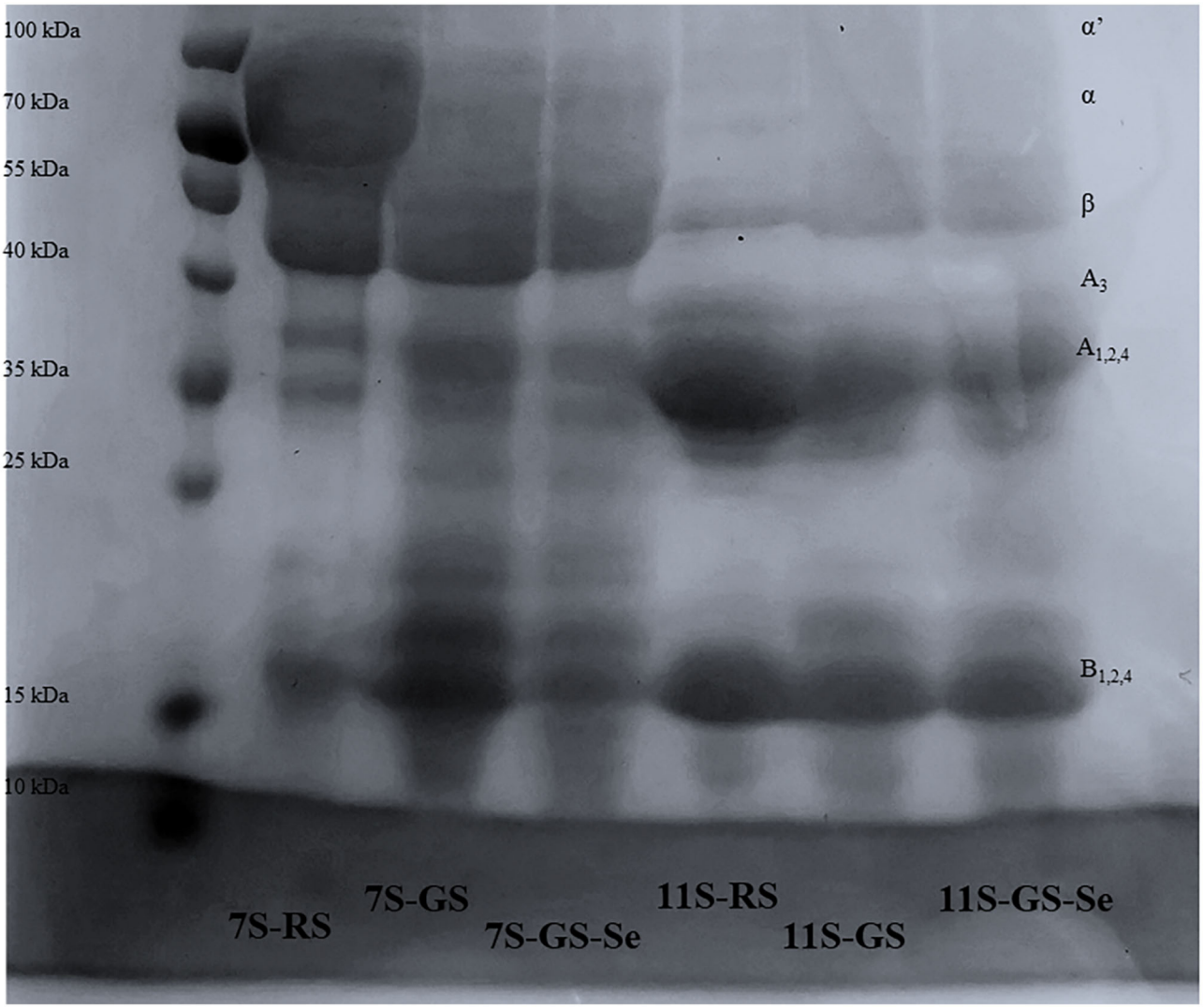

FIGURE 3 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of different soybean proteins.

the results were consistent with those of a study on soybeans cultivated on farms. Deng et al. (7) found that the concentrations of Se in 11S are significantly higher (38.6%) than those in 7S; Wang et al. (38) also found that the concentration of Se in 11S is considerably higher (12.1%) than that in 7S in natural Se-enriched raw soybeans in Enshi, indicating that the 11S can bind Se more efficiently than 7S. However, Zhao et al. found that the Se content of 7S and 11S from Se biofortification soybeans is about 9.9-fold higher compared with ordinary soybeans, and there is no significant difference between 7S and 11S (25), which may be due to differences in the methods of soybean cultivation, extraction, and analysis. Above all, the ability of 11S to enrich Se in raw soybeans compared that of 7S was not greater than that of Se-enriched soybeans during germination, indicating that it is much easier to combine Se with 11S than with 7S during germination.

SeMet and MeSeCys were the dominant Se species in the germinated soybeans, indicating that selenite is easily converted to organic species during germination and can be efficiently incorporated into proteins (39). The decreased proportion of organic Se with increasing Se treatment concentrations was in line with a previous study, in which the proportion of organic Se in plants decreased from 88 to 80%, indicating that an increased concentration of Se in fertilizer will reduce the efficiency of conversion from inorganic to organic Se (30). Similar to the results for the total Se contents, the concentrations of different species were far higher in 11S than those in 7S. A plausible reason for this phenomenon is that S-containing amino acids in 7S were lower than those in 11S, and a previous study found that Se is incorporated into proteins mainly through taking the place of S in the S-containing amino acids (7). Previous studies also found that Se is mainly incorporated into proteins with low-molecular-weight compounds, such as in selenium-enriched mushrooms (not more than 16 kDa), soybean (15–20 kDa), Se-enriched rice (<36.3 kDa), and Se-enriched *Tenebrio molitor* larvae (<40 kDa)

TABLE 3 | Secondary structural compositions of different proteins (%).

Protein	α-Helix	β-Sheet	β-Turn	Random coil
7S-RS	15.26 ± 0.77 c	44.55 ± 3.37 a	20.35 ± 0.84 b	19.84 ± 3.31 a
7S-GS	21.14 ± 2.12 a	40.75 ± 3.54 ab	22.85 ± 1.08 a	19.42 ± 0.85 a
7S-GS-Se5	17.49 ± 0.56 bc	40.73 ± 0.01 ab	22.06 ± 0.09 ab	20.07 ± 0.02 a
7S- GS-Se30	19.02 ± 1.00 ab	39.02 ± 1.34 b	22.23 ± 0.92 ab	19.73 ± 0.60 a
7S- GS-Se60	16.64 ± 0.94 c	40.42 ± 1.62 ab	23.52 ± 2.16 a	19.42 ± 0.39 a
11S-RS	21.43 ± 0.51 a	43.68 ± 1.16 a	22.52 ± 1.82 b	12.36 ± 0.15 c
11S-GS	17.13 ± 0.67 b	39.51 ± 1.50 bc	21.73 ± 2.01 b	21.63 ± 0.17 a
11S- GS-Se5	18.09 ± 0.72 ab	38.71 ± 0.41 c	21.98 ± 0.67 b	21.22 ± 0.98 a
11S- GS-Se30	15.92 ± 1.07 b	41.35 ± 0.73 ab	23.55 ± 0.81 ab	19.42 ± 2.95 ab
11S- GS-Se60	16.10 ± 3.82 b	38.99 ± 0.60 bc	27.95 ± 2.72 a	16.96 ± 1.69 b

Different letters are used to show significant differences at various treatments ($p < 0.05$).

(11, 40–42). The contents of low-molecular-weight subunits in 11S were higher than those in 7S, and this may also be the reason for higher Se content in 11S.

Effects of Se on Amino Acids and Protein Structure

Germination had no significant effects on most of the amino acids, with amino acid contents increasing slightly without significant differences; however, germination led to a significant increase in the total amino acids. Yang et al. found that the germination process leads to a significant decrease in some amino acids (23). However, Gao et al. found that germination increased the amino acid concentration of soybeans (22). The differences between the previous results and our work could be due to differences in the varieties of soybean and conditions during germination. Se treatment had no significant effect on amino acids but decreased methionine in 11S. A similar phenomenon has also been reported by Zhao et al. (25), who found that Se had no significant effect on the concentrations of most amino acids in raw soybean and only caused a reduction in concentrations of cysteine and methionine. This result could be due to Se taking the place of S in these two amino acids so that parts of methionine and cysteine are converted to SeMet and SeCys (43).

The subunits of 7S and 11S extracted from RS, GS, and GS-Se samples were analyzed by SDS-PAGE, and there were no new bands formed and existing bands did not disappear, indicating that Se biofortification did not make protein subunits degrade or aggregate. However, 7S and 11S were partially degraded into low-molecular-weight peptides due to germination, which is consistent with previous research (23, 44). Previous studies have found that 7S and 11S share the same bands between Se-enriched and ordinary raw soybeans (7, 11, 25). These studies indicate that the Se incorporated into proteins does not significantly affect the subunit composition of soybean proteins. However, Luo et al. (30) found that as Se fertilizer increases to 100 g/ha, the protein subunit of molecular weight of ∼30 kDa moves upward, because the high Se content leads to protein subunit binding. The different phenomena observed in these studies may be due to the concentration of Se. A low concentration of Se does not change the protein subunit distribution, and a high concentration of Se can influence the molecular weight of proteins.

Based on the results detailed in **Table 3**, it can be concluded that Se influenced the secondary structures of proteins to some extent. Se is bound to amino acids and then incorporated into soybean proteins, which may influence the protein structure and also the secondary structure (17). It has been speculated that the decrease in the concentration of Cys and Met is caused by the replacement of S with Se in these two amino acids, both being hydrophobic (30). If Se converts S–S into Se–Se, indicating that the disulphide bond has changed, together with the atomic size and ionization of Se, then the secondary structure will be influenced (45). Zhao et al. (25) speculated that Se could influence the secondary structures of 7S and 11S in raw soybeans. However, Deng et al. (7) found a different phenomenon that Se has no significant effects on the secondary structure of 7S and 11S in raw soybean. It is possible that the interference of Se species with the contents of sulfhydryl groups and disulfide bonds is negligible because the Se content in the protein is much lower than that of S (30). Therefore, the specific reasons for the effect of Se on protein secondary structure need to be further investigated.

Effects of Se on Different Protein Functional Properties

The improvement in protein WHC of 7S and 11S by germination was consistent with the results of SDS-PAGE experiments, in which 7S and 11S are hydrolysed by enzymes during germination (44), and the high WHC of proteins could be attributed to the high hydrophilicity of the soybean proteins (3). It seems that with the increased grade of hydrolysis, the protein may easily hold more water, and there is a higher WHC. The present study found that Se had no significant effect on the WHC and OHC, which is consistent with the study conducted by Deng et al. (7). However, Lazo-Velez et al. found that germinated soybeans enriched with Se promote WHC (46). The water-holding ability is an important functional property in food processing, such as in the preparation of sausages, pumped meats, and confections, as it increases product yields (47). Higher oil absorption can be useful for ingredients in meat, sausages, and dairy products, where the oil-holding ability affects the texture of the food (48).

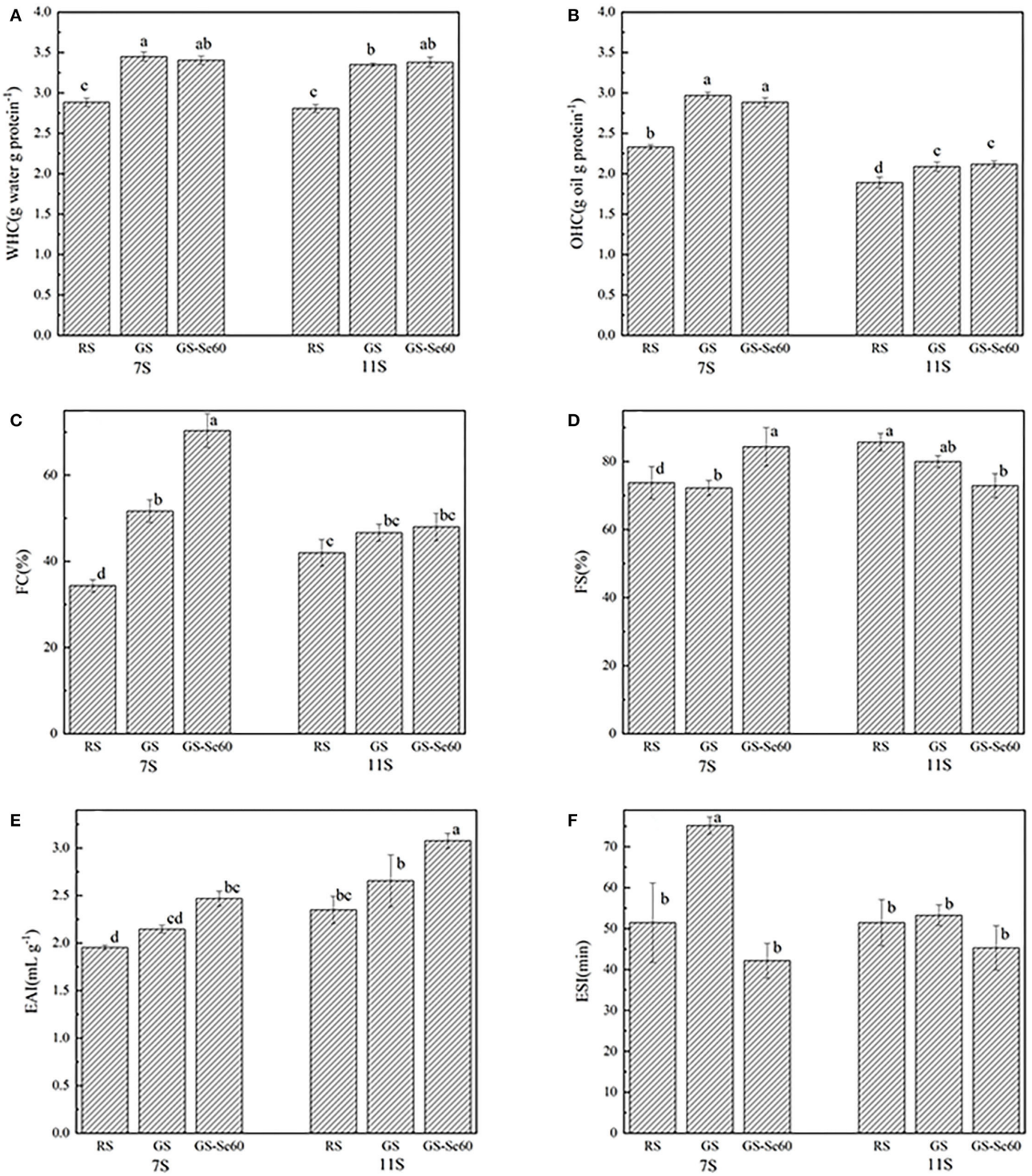

FIGURE 4 | Functional properties of different soybean proteins treated with or without Se. **(A)** Water holding capacity (WHC), **(B)** oil holding capacity (OHC), **(C)** foaming capacity (FC), **(D)** foam stability (FS), **(E)** emulsion activity index (EAI), and **(F)** emulsifying stability index (ESI). Different letters are used to show significant differences at various treatments ($p < 0.05$).

Germination increased the FC of 7S and 11S and decreased the FS, which is consistent with previous studies (26, 49). This may be because the increased concentration of polypeptide, which is produced during soybean germination, could promote FC by incorporating more air (23). According to a previous study, a reduction in FS may be due to the low strength of micropeptides

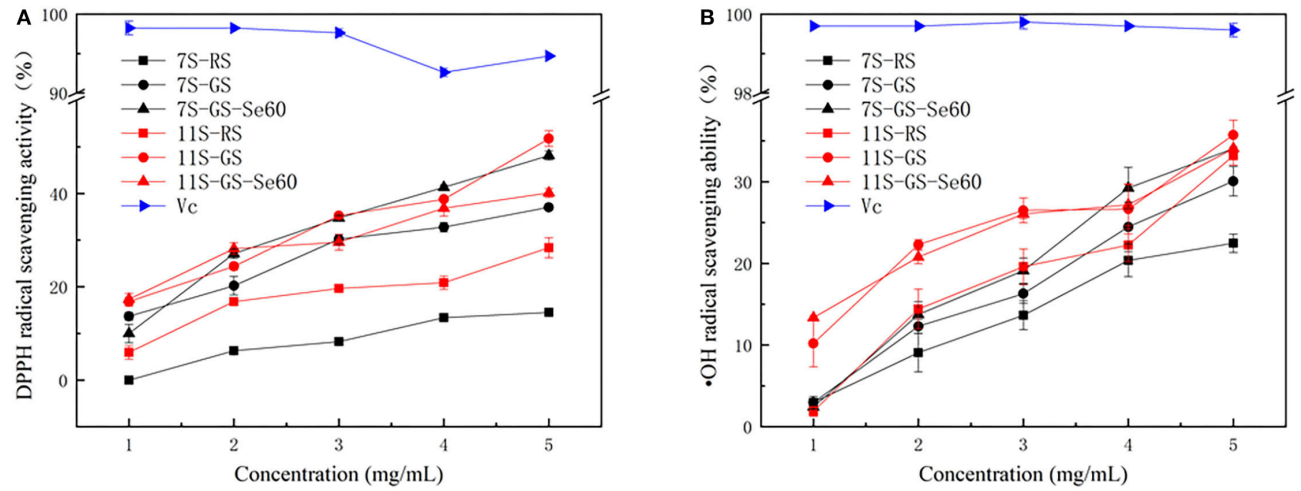

FIGURE 5 | 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity **(A)** and hydroxyl free (·OH) radical scavenging ability **(B)**.

that maintain the stability of the foam (49). Therefore, the increased FC and decreased FS of GS in this study could be caused by the high solubility and low molecular weight of peptides. These low-molecular-weight peptides do not promote stable foams because of the reduced interactions between proteins (26, 50). The low FS is caused by weak interfacial surface films between bubbles, and the bubbles tend to collapse (51). The FC and FS of protein are important for processing cakes, whipped desserts, and ice cream (47). Interestingly, the 7S of GS-Se had both high FA and FS, and studies have shown that the FS depends on the rheological properties of the protein-membrane, as well as the protein-protein interactions and environmental factors (52). Thus, this result may be due to Se producing greater electrostatic repulsion, thus stabilizing the foams.

Previous studies have reported that the composition and structure of protein would influence emulsifying properties (53). In the present study, germination increased the EAI and ESI of 7S and 11S, and Se treatment increased EAI but decreased ESI. A similar phenomenon in germination was found in some previous studies, in which EAI and ESI were influenced by germination time (22, 44). Yang et al. found that germination can significantly increase the EAI of soybean protein but has no effect on ESI (23). Enzymatic hydrolysis could promote EAI and ESI by increasing the solubility of protein, which is important for emulsifying properties (26). A high solubility allows proteins to rapidly diffuse and adsorb at the interface between water and oil (3). Germination improved the EAI and ESI compared with those of raw soybean, which may also be caused by the increased solubility, so proteins can migrate to the interface rapidly (54). Overall, the changes in the protein content, amino acid composition, and protein structure might affect the functional properties of soybean proteins (17).

Effects of Germination and Se on the Antioxidant Capacity

Previous studies have shown that germination can improve the antioxidant capacity of many products, such as soybean (55), kale, kohlrabi (56), and wheat (57), but these studies have mainly focused on the whole product powder; they attributed the improved antioxidant capacity to phenolics and flavonoids. This has also been verified by other studies, where higher antioxidant capacities are observed in germinated soybean mainly because of an increase in the concentration of total isoflavones (58, 59). Gao et al. found that soybean sprout protein effectively eliminates DPPH and •OH free radicals, showing that germination can strengthen the antioxidant activity of soy proteins (22). In the present study, we further investigated 7S and 11S, the two main protein fractions, and the antioxidant capacities of both proteins were improved through germination; the plausible reason is that germination can transform protein into small peptides, which have strong radical-scavenging abilities (60).

Selenium improved the ability of 7S to scavenge DPPH-free radicals and hydroxyl radicals in our study, which has also been found in previous studies (12, 61, 62); Se-containing proteins exhibit significantly higher antioxidant ability *in vitro* than proteins without Se (63). Se can promote both enzymatic and non-enzymatic antioxidant systems, such as GPx and glutathione (64). Se also increases some hydrophobic amino acid content in proteins, thereby enhancing the antioxidant capacity (40). A diselenide bridge, formed during the oxidation of two neighboring Cys residues, is longer than a disulfide bridge and has a lower redox potential (65). Therefore, a plausible reason is that 7S-containing Se–Se bonds tend to have higher antioxidant capacity than that containing S-S bonds.

However, Se had opposite effects on the antioxidant activities of 11S in our study, which cannot be reasonably explained, as the antioxidant mechanism of Se-enriched soy protein may be related to one or more of these mechanisms. Antioxidant properties are promoted with an increased concentration of Se in proteins (11). The antioxidant ability is affected not only by Se content and species but also by other factors, such as protein and amino acids (66), or by reducing sugars, ascorbic acid, and organic acids, among others, which may influence the evaluation of the antioxidant ability (64). Because the

antioxidant activity is influenced by so many factors, the specific mechanism by which Se enhances the antioxidant activity of the protein is still uncertain, and the changes in antioxidant activity need to be further studied to clarify how Se affects the antioxidant activity. In addition, *in vitro* experiments are widely used to evaluate antioxidant capacity. However, these reactions are not just involved in the antioxidant enzymes in organisms, and evaluation methods should simulate real physiological conditions in organisms or involve tests using animal models.

CONCLUSION

SeMet and MeSeCys were the main Se species in the germinated soybeans, and dominant proteins 7S and 11S had different Se enrichment abilities. Se treatment had no significant effects on amino acids but decreased methionine in 11S. Moreover, the contents of α-helix decreased with increasing Se concentration, while the other structures were not significantly different. Se treatment had no significant effects on WHC and OHC but increased the FC and EAI of 7S, but only the EAI of 11S. Furthermore, Se treatment increased the antioxidant capacity in 7S but had no significant effects on that of 11S. The present study provides initial insight into the Se distribution in different germinated soybean dominant proteins and its effects on protein structure, functional properties, and antioxidant activity. The results provide important evidence for the development of efficient natural Se-enriched food supplements and the processing of Se-enriched germinated soybean protein.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YH contributed to methodology, study design, and manuscript writing. NL, YX, and YL investigated the study. BF and LT contributed to data analysis. FW, CB, and PM contributed to study design, manuscript review, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.849928/full#supplementary-material

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Selenium Effect Threshold for Soil Nematodes Under Rice Biofortification

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Crop biofortification with inorganic selenium (Se) fertilizer is a feasible strategy to improve the health of residents in Se-deficient areas. For eco-friendly crop Se biofortification, a comprehensive understanding of the effects of Se on crop and soil nematodes is vital. In this study, a rice pot experiment was carried out to test how selenite supply (untreated control (0), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, or 200 mg Se kg^{-1}) in soil affected rice growth, rice Se accumulation, and soil nematode abundance and composition. The results showed that selenite supply (5–200 mg kg^{-1}) generally increased the number of rice tillers, rice yield, and Se concentrations in rice grains. In soil under 10 mg kg^{-1} Se treatment, the genus composition of nematodes changed significantly compared with that in the control soil. With increased Se level (> 10 mg kg^{-1}), soil nematode abundance decreased significantly. Correlation analysis also demonstrated the positive relationships between soil Se concentrations (total Se and bioavailable Se) with rice plant parameters (number of rice tillers, rice yield, and grain Se concentration) and negative relationships between soil Se concentrations (total Se and bioavailable Se) with soil nematode indexes (nematode abundance and relative abundance of *Tobrilus*). This study provides insight into balancing Se biofortification of rice and soil nematode community protection and suggests the effective concentrations for total Se (1.45 mg kg^{-1}) and bioavailable Se (0.21 mg kg^{-1}) to soil nematode abundances at 20% level (EC20) as soil Se thresholds. At Se concentrations below these thresholds, rice plant growth and Se accumulation in the grain will still be promoted, but the disturbance of the soil nematodes would be negligible.

Keywords: selenium, threshold, rice, rhizosphere, biofortification, nematodes

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INTRODUCTION

Selenium (Se) is an essential element for humans and other animals. It contributes to the protection of liver function and antioxidant defense systems (Brown and Arthur, 2001). Keshan disease and Kashin–Beck disease have been closely linked to low Se intake in humans (Combs, 2001; Fairweather-Tait et al., 2011). Se has been reported to improve symptoms of viral infections,

cardiovascular disease, and cancer (Rayman, 2000). The distribution of Se in the soil is uneven. In the Se-rich areas of Ziyang and Enshi in China, the Se concentration in soil reached up to 36.69 and 86.59 mg kg^{-1}, respectively (Dinh et al., 2018; Li et al., 2020a). Meanwhile, Se-deficient regions traversing the northeastern region of China until the eastern region of the Tibetan plateau were reported with Se concentrations below 0.20 mg kg^{-1} (Dinh et al., 2018). To obtain enough Se from food, inorganic Se fertilizer has been applied to soil to increase crop Se concentration and hence to overcome the problem of inadequate Se intake by residents in China (Wang et al., 2013a; Wu et al., 2015). However, Se overfertilization is occurring in Se-deficient soils (Winkel et al., 2015; Ros et al., 2016). Excessive Se flux into the soil as a consequence of Se biofortification may exert negative influences on the soil fauna since they remain in the soil for their entire life cycle and are directly affected by the chemicals in the soil (Xu et al., 2022).

Despite the increasing information available on the effects of Se on plants (Lin et al., 2005; Cabral Gouveia et al., 2020), mammals (Benko et al., 2012), and microorganisms (Mojtaba et al., 2015), less is known about the effects of Se on soil fauna. Nematodes are the most abundant metazoans in soil ecosystems, and they are directly involved in the accumulation of organic matter and nutrient cycling (Paz-Ferreiro and Fu, 2016). With more attention to their ecological significance, soil nematodes have been used increasingly as indicators in monitoring soil ecosystem quality (Bongers and Ferris, 1999; Ekschmitt et al., 2001; Neher, 2001). Nematode abundance and composition have been shown to accurately reflect the disturbance caused by fertilizers and heavy metals in soil ecosystems (Bongers et al., 2001; Georgieva et al., 2002; Chen et al., 2003; Zhang et al., 2006; Park et al., 2016).

Limited studies revealed the detrimental effect of Se on soil nematodes. For example, Bakonyi et al. (2003) found that nematode abundance and the number of nematode genera in the experimental group (soil with an ammonium acetate EDTA-extracted Se concentration of 11 mg kg^{-1}) were significantly reduced compared with the control group. Se-induced changes in soil nematodes were attributed to omnivorous and predatory nematodes, which could respond quickly to the high-Se stress. In another study (Prins et al., 2019), selenate treatment (80 μM, twice a week) significantly decreased nematode abundance in the rhizosphere soil of the Se-hyperaccumulator plant *Stanleya pinnata*. However, Se was only regarded as a pollutant in the above studies. No study has been conducted yet to reveal the effect of Se on soil nematodes in a soil–plant system under Se biofortification.

Considering the multiple effects of Se (Lv et al., 2021), we hypothesized that soil nematodes will not be disturbed under biofortification with a small amount of Se, while excessive Se may harm soil nematodes and affect plant growth. To test this hypothesis, a rice pot experiment with selenite supplementation at different concentrations was carried out. The objectives were (1) to study the effects of selenite supply on rice plant growth and Se accumulation in the grain, (2) to evaluate the effect of selenite application on soil nematodes, and (3) to determine the soil Se concentration threshold based on nematode response to Se supply.

MATERIALS AND METHODS

Pot Experiment and Plant Growth

A pot experiment using rice was carried out in a greenhouse at the Guangxi Academy of Agricultural Sciences, China, from August to December 2019. The rice cultivar used was *indica* rice Baixiang139, and the rice seedlings germinated and cultured in an incubator under constant temperature (30°C) and light (14 h day^{-1}) for 3 weeks were provided by the Guangxi Academy of Agricultural Sciences, China. In 2019, fresh paddy soil was collected from a field in Guigang City, Guangxi. The characteristics of the soil were determined according to the methods of Liu et al. (2018) and are recorded in **Table 1**. To keep the native nematodes in the soil, fresh soil was used in the pot experiment instead of dry soil. Specifically, the collected fresh soil was broken into small pieces and stirred by a wooden spoon to make it as homogenized as possible. Each pot (diameter: 28.5 cm and height: 27.5 cm) was loaded with 8 kg of fresh weight homogenized soil. Sodium selenite (Na_2SeO_3, the major component of Se fertilizer) was used in preparation for a 10 g Se L^{-1} concentrated solution. Then the diluted Se solution (2 L) or ultrapure water (2 L) were added into the pots to attain soil Se concentrations of 0 (untreated control, CK), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, or 200 mg Se kg^{-1} soil, respectively. The experimental Se concentration range of 0–200 mg kg^{-1} was designed based on the wide soil Se range worldwide, especially the Se-rich hotspots in China, like Enshi in Hubei Province, Ankang in Shaanxi Province, Yichun in Jiangxi Province (Dinh et al., 2018), and overuse of Se fertilizer in Se-deficiency soils (Winkel et al., 2015; Ros et al., 2016). Moreover, the similar wide ranges of Se levels in previous studies were also taken into consideration in this study (Kuperman et al., 2018; Xiao et al., 2018). Three replications were set up at each concentration, with a total of 39 pots. Compound fertilizer (2 g kg^{-1}, N:P_2O_5:K_2O ratio of 14:12:14) was added to each pot. After 20 days of aging (Li et al., 2016), three rice seedlings (16 cm, 3 leaves) were planted into each pot. The pots were placed in the greenhouse under natural conditions of illumination and temperature. Se-free water was used for irrigation to simulate flooded paddy conditions. Waterline 4 cm above the soil surface in each pot was set for water content controlling in the whole growth period. After 4 months, mature rice plants were uprooted, and the rhizosphere soil attached to the root surface was collected from each pot carefully (Breidenbach et al., 2016) for later nematode analysis. The rest of potting soil was also collected for Se analysis. The rice plants were washed with deionized water and air-dried. The height of the main culm and the number of tillers were recorded. The grains were separated from plants and dried in an oven at 60°C for 16 h to determine the yield (the dry weight of grains per rice plant).

Soil and Grain Se Analysis

The potting soil was air-dried and homogenized to pass through a 100-mesh sieve. Grains were dehulled, polished, and ground

TABLE 1 | Physicochemical properties (dry weight) of the paddy soil used in this study.

Se concentration (mg kg^{-1})	Total nitrogen (g kg^{-1})	Total phosphorus (g kg^{-1})	Total potassium (g kg^{-1})	Hydrolysable nitrogen (mg kg^{-1})	Available phosphorus (mg kg^{-1})	Available potassium (mg kg^{-1})	Organic matter (g kg^{-1})	pH
0.42	1.49	0.92	10.9	60.7	20.3	63.2	21.0	7.05

The contents of nitrogen, phosphorus, and potassium represent the contents of elements N, P, and K.

to pass through a 100-mesh sieve. The total Se concentrations in soil or grain were determined using the method described by Long et al. (2020). Briefly, samples (0.2 g soil or grain) were added to a conical flask and then digested for 12 h with 8 ml nitric acid and 2 ml perchloric acid at room temperature. The digested solution was heated on an electric heating plate until white fumes were observed. After cooling, the conical flask walls were rinsed with deionized water, and the solution in the flask was concentrated by reheating until 2 ml solution was left. An aliquot (5 ml) of 12 mol L^{-1} HCl was added to the sample solution to reduce selenate to selenite. Se concentration was determined using the hydride generation atomic fluorescence spectrometry (HG-AFS). The detection limit of this method of Se detection is 0.08 μg kg^{-1}. Bioavailable Se in soil was extracted with 0.1 mol L^{-1} KH_2PO_4 (Zhao et al., 2005), then the total Se in the supernatant was determined by HG-AFS. National standard reference materials Bush Branch (GBW 07603-GSV-2, Se = 120 ± 20 μg kg^{-1}) and Chestnut Soil (GBW 07402-GSS2, Se = 160 ± 40 μg kg^{-1}) were used to check the accuracy of Se detection, and the recovery rates ranged from 93.7 to 106.2% in this detection.

TABLE 2 | Total and bioavailable Se concentration (dry weight) in the soil after plant harvest.

Nominal Se concentration (mg kg^{-1})	Total Se concentration (mg kg^{-1})	Bioavailable Se concentration (mg kg^{-1})
0 (CK)	0.42 ± 0.06	0.04 ± 0.01
5	7.90 ± 0.51	1.33 ± 0.08
10	9.25 ± 2.35	1.88 ± 0.37
15	14.33 ± 1.78	1.85 ± 0.35
20	21.08 ± 1.31	2.62 ± 0.36
25	25.80 ± 9.52	2.90 ± 0.52
30	27.77 ± 2.54	2.42 ± 0.38
35	30.72 ± 3.35	3.14 ± 0.44
40	32.50 ± 6.83	2.61 ± 0.56
45	46.47 ± 3.48	3.05 ± 0.80
50	57.80 ± 6.21	4.13 ± 0.43
100	135.74 ± 11.99	4.70 ± 0.94
200	196.29 ± 4.38	6.37 ± 0.75

Isolation and Analysis of Soil Nematodes

Nematodes were isolated from the rhizosphere soil samples using the sucrose centrifugal flotation method (Li et al., 2020b). In brief, 100 g soil and 100 ml water were added into a 250-ml centrifuge tube, and a glass rod was used to stir the sample thoroughly. The suspension was centrifuged (810 × *g* for 5 min), and then the supernatant was discarded while the sedimentary soil containing nematodes was retained. An aliquot (100 ml) of sucrose solution (454 g L^{-1}) was added to the tube to re-suspend the sediment, and the suspension was centrifuged again (280 × *g* for 5 min). The supernatant containing nematodes was passed through two 500-mesh sieves, then the nematodes retained in the sieves were collected, counted, and identified to the genus level (Bongers, 1988), using an inverted compound optical microscope. Based on trophic type, nematodes were divided into five trophic taxa: bacterivores, algivores, fungivores, herbivores, and omnivores–predators (Yeates et al., 1993). Based on life strategy, nematodes were assigned to five taxa with colonizer–persister (c-p) values ranging from 1 to 5 (Ferris et al., 2001).

Statistical Analysis

All data represent the means ± standard deviations (SD) of three replicates for each treatment. One-way ANOVA analysis with Tukey multiple range tests for *post hoc* mean comparisons was carried out to identify significant differences ($p < 0.05$) among the different Se treatments. Pearson's correlation analysis was performed to verify the correlations between soil Se concentrations (total Se and bioavailable Se) with indexes of rice plants and soil nematodes. Parametric non-linear regression analysis was used to quantify the relationships between soil Se concentrations (total Se and bioavailable Se) with nematode abundances. The no observed effect concentration (NOEC) was identified as the highest Se concentration showing a response not significantly compared with CK, and the effective concentration at the level of 20% (EC20) was identified as the Se concentration producing a 20% decrease in the measured parameter compared with CK (Kuperman et al., 2018). SPSS 25.0 (IBM, Armonk, NY, USA) was used for statistical analysis, and Origin 2021b (OriginLab, Northampton, MA, USA) was used for visualization.

RESULTS

Total and Bioavailable Se Concentration in Soil

The total Se concentrations and bioavailable Se concentrations of potting soils are shown in **Table 2**. The total Se concentrations were close to nominal Se concentrations, and the bioavailable Se concentrations increased with the elevated total Se concentrations.

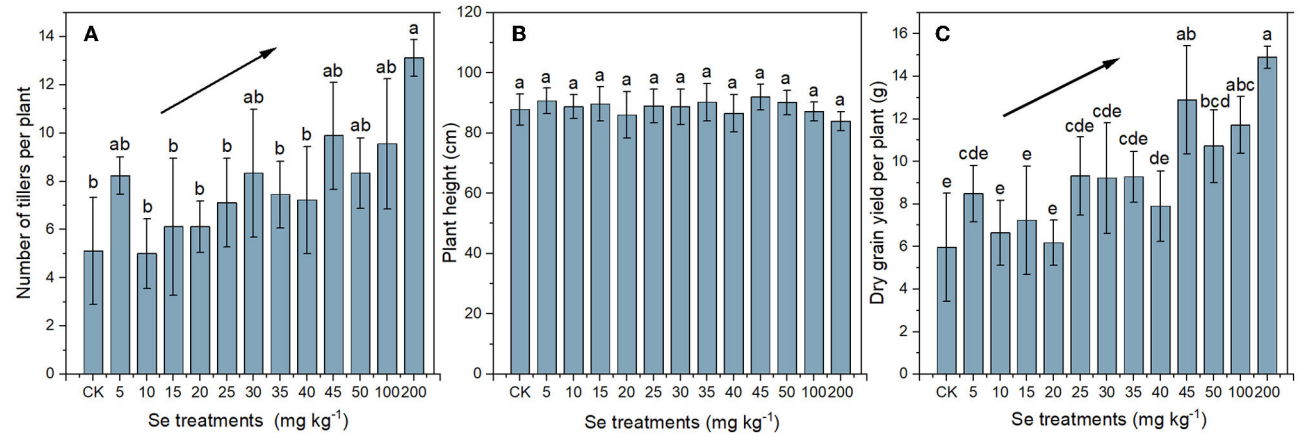

FIGURE 1 | Number of tillers per plant **(A)**, height of the main culm **(B)**, and yield per plant **(C)** of rice plants under different Se treatments. The black lines with arrows indicate increasing trends. Values represent mean ± SD. Any two samples within a bar chart sharing a common letter are not significantly different at the $p < 0.05$ level.

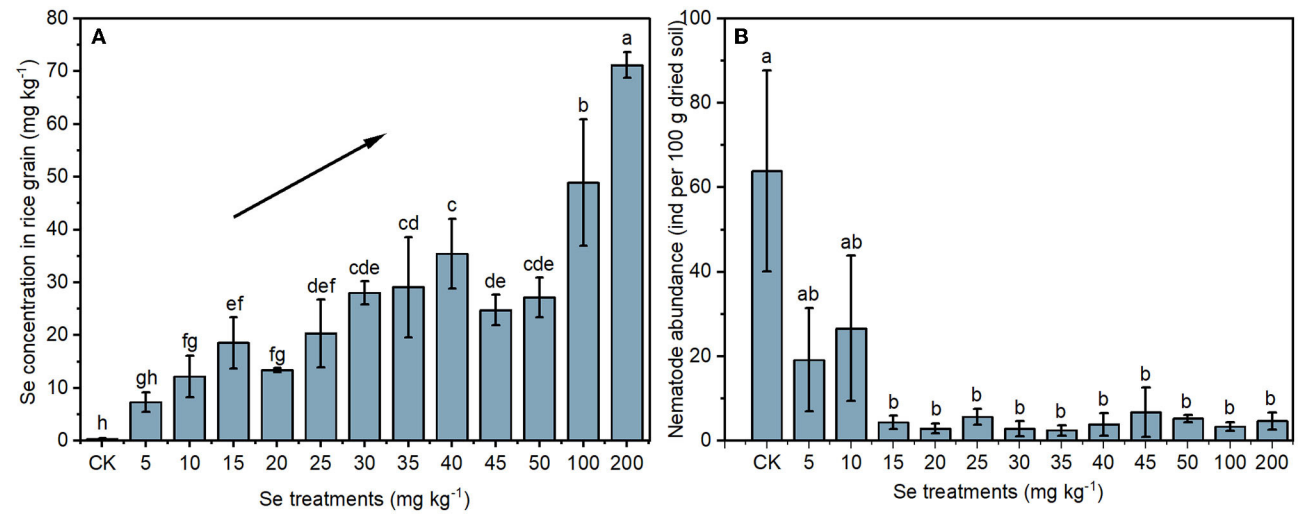

FIGURE 2 | Selenium concentrations in rice grains **(A)** and the abundance of nematodes in soil **(B)** under different Se treatments. The black line with an arrow indicates an increasing trend. Values represent mean ± SD. Any two samples within a bar chart sharing a common letter are not significantly different at the $p < 0.05$ level.

Rice Plant Growth and Se Accumulation

Plant Growth

The number of tillers, height of the main culm, and grain yield per plant of rice under different Se treatments are shown in **Figure 1**. Compared with the CK, no significant difference in tillering was found in rice under low Se treatments (≤100 mg kg^{-1}). However, the tillers number increased to 13.11 ± 0.78 under 200 mg kg^{-1} Se treatment, which was 2.57 times as much as that of CK (5.11 ± 0.22) (**Figure 1A**). The mean height of the mature rice plants ranged from 83.89 to 91.89 cm, and there was no significant difference in height of plants under different Se treatments (**Figure 1B**). Variation in grain yield in relation to Se treatment shared a similar trend with tillering variation, and the maximum yield (14.89 ± 0.52 g $plant^{-1}$) was exhibited in rice under 200 mg kg^{-1} Se treatment, being 2.50 times that of the CK yield (5.96 ± 2.54 g $plant^{-1}$) (**Figure 1C**). In general, the number of tillers and rice yield increased with the increase of supplied Se, indicating that the selenite supply promoted rice growth.

Se Concentration in Rice Grain

The Se concentrations in rice grains under different Se treatments are shown in **Figure 2A**. Without Se supply, the Se concentration in CK rice grain was 0.31 ± 0.22 mg kg^{-1}. Se concentrations in rice grains increased after Se supply, and the highest concentration of 71.17 ± 2.43 mg kg^{-1} was detected in rice with a 200 mg kg^{-1} Se supply. In general, rice grain Se concentrations increased with the increase of supplied Se.

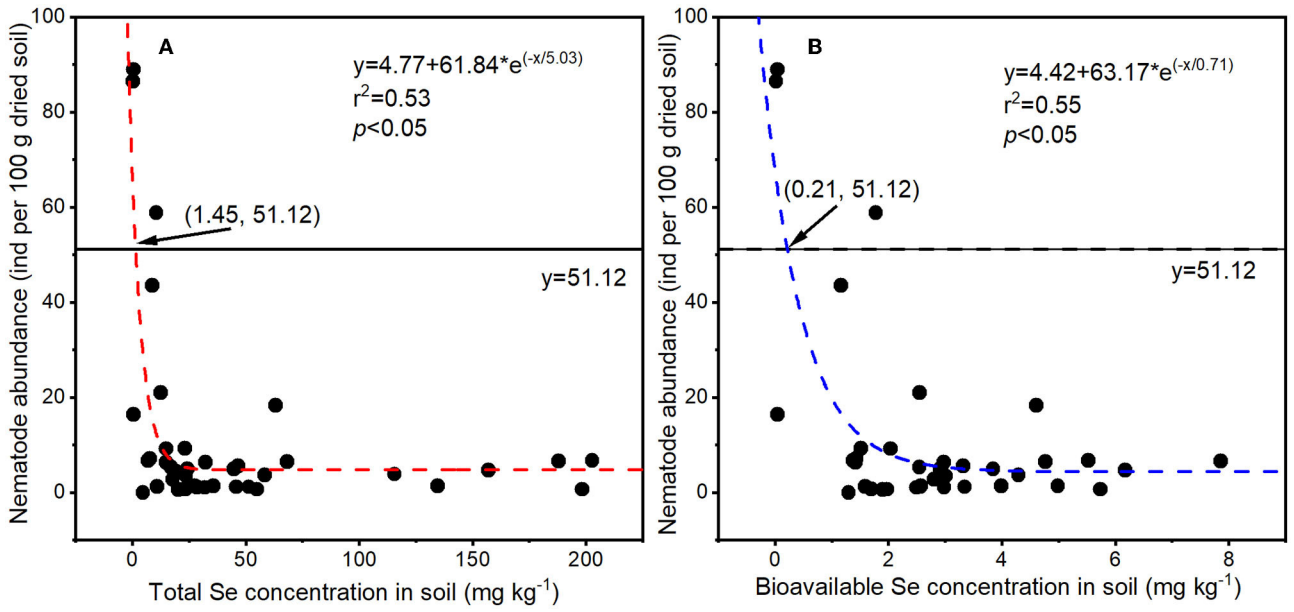

FIGURE 3 | Relationships between soil Se concentrations [total Se **(A)** and bioavailable Se **(B)**] with nematode abundances ($n = 39$). The straight $y = 51.12 = 63.90 \times 80\%$, 63.90 was the nematode abundance in CK soil, 51.12 was the nematode abundance corresponding to EC20, and 1.45 and 0.21 were EC20 for total Se and bioavailable Se in soil nematode abundance.

Soil Nematode Abundance and Composition

Nematode Abundance

To determine the effect of selenite on soil nematodes, the nematode abundance and composition were analyzed. As shown in **Figure 2B**, soil nematode abundance (number of nematodes in 100 g of dried rhizosphere soil) in the CK group was 63.90 ± 23.79. The nematode abundance in rhizosphere soil under 5 or 10 mg kg^{-1} Se treatment was lower than that in the CK, though not significantly. However, a significant reduction in nematode abundance was observed in soil under higher Se treatments (> 10 mg kg^{-1}). The NOEC for total Se and bioavailable Se to soil nematode abundance were therefore 9.25 and 1.88 mg kg^{-1} (actual soil Se level under 10 mg kg^{-1} Se treatment), respectively. Parametric non-linear regression analysis was used to quantify the relationships between actual soil Se concentration (total Se and bioavailable Se) with nematode abundance (**Figure 3**). The EC20 for total Se and bioavailable Se to nematode abundance were 1.45 and 0.21 mg kg^{-1}, respectively.

Genus Composition of the Soil Nematodes

A total of 30 nematode genera were detected in all samples, with 15 genera being quite common (relative abundance ≥ 5%). The relative abundance of individual nematode genera is presented in **Figure 4**, with the rarer 15 genera being classified into one group (others). The genus composition of the soil nematode community varied in soil under different Se treatments. With 5 mg kg^{-1} Se supplement, the soil nematode community exhibited a genus distribution similar to that of the CK in which the predominant genus was *Tobrilus*. The NOEC for soil total Se and bioavailable Se to genus composition of nematodes were therefore 7.90 and 1.33 mg kg^{-1} (actual soil Se level under 5 mg kg^{-1} Se treatment), respectively. Under higher Se treatments (10, 15, or 20 mg kg^{-1}), the genus distribution of the nematodes changed, and the predominant genera became *Panagrolaimus* and *Rhabdolaimus*. As the Se concentration increased furtherly (>20 mg kg^{-1}), a smaller proportion of algivores and a greater proportion of herbivores were observed, with *Scutylenchus*, *Dolichorhynchus*, and *Hirschmanniella* being the predominant genera. Overall, excess selenite supplementation shifted the composition of the nematode community from an algivore-dominated one to an herbivore-dominated community and exerted stress on *Tobrilus*.

Taxa Composition of the Soil Nematodes

The relative abundance of soil nematode taxa, classified by trophic type or cp-value, is shown in **Table 3**. With respect to trophic type, the bacterivores, algivores, and herbivores were the dominant taxa in all groups, whereas the fungivores and omnivores–predators were less frequent. In the CK soil, the relative abundances of algivores and herbivores were 69.17 ± 10.42 and 4.39 ± 4.45%, respectively. However, under 200 mg kg^{-1} Se treatment, the relative abundances of algivores and herbivores were 7.41 ± 12.83 and 81.48 ± 32.08%, respectively. Confirming the findings from the genus composition, nematode community composition shifted toward an herbivore-dominated community as the supplied Se concentration increased. With respect to cp-value taxa, the cp-3 nematodes were the predominant taxa in all treatment groups. There was no significant

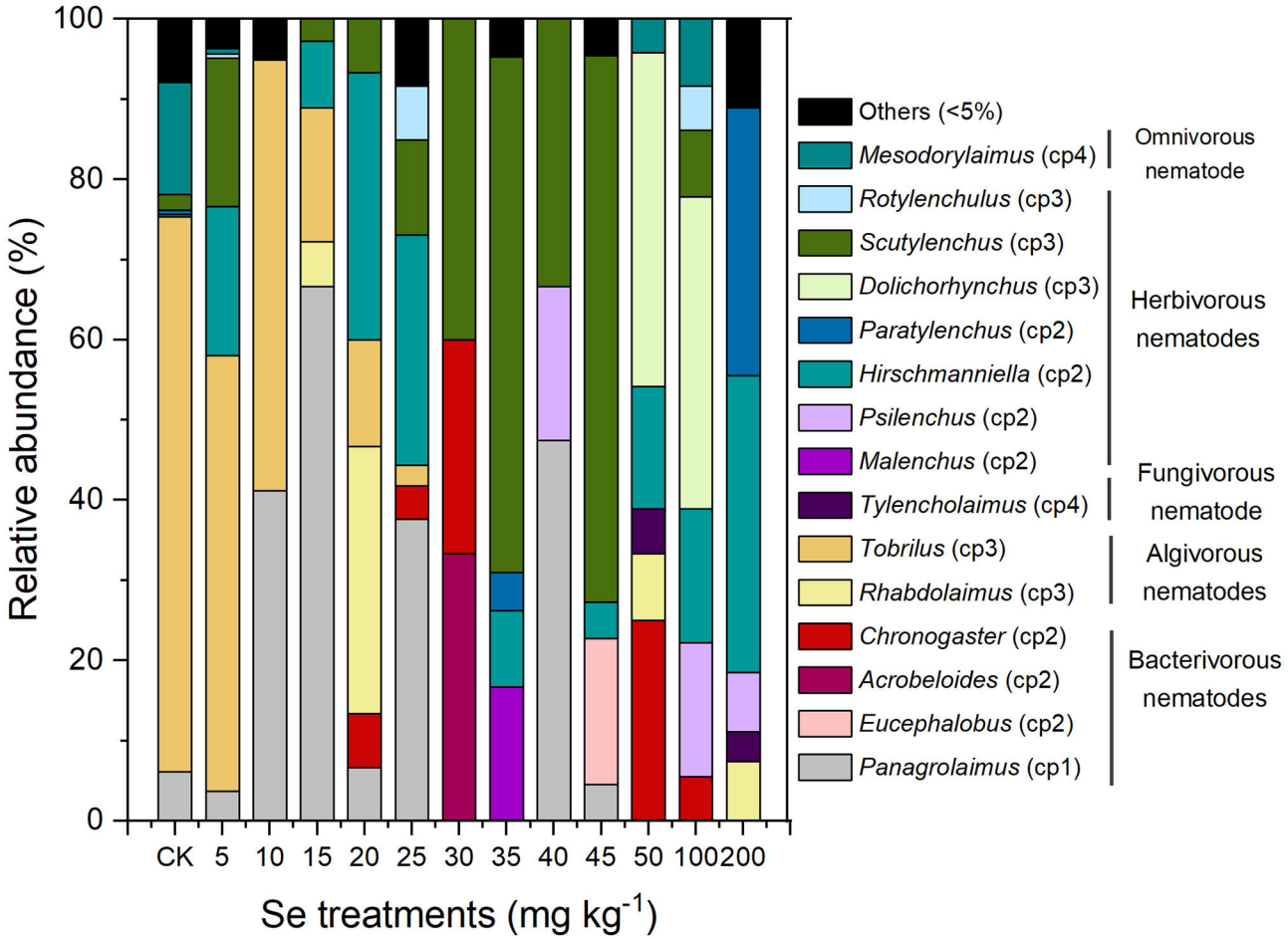

FIGURE 4 | Genus composition of the nematodes in soil under different Se treatments.

difference in relative abundances of the cp-3 taxon in different treatment groups.

DISCUSSION

The Effects of Selenite on Rice Plant Growth and Se Accumulation

In this study, the number of tillers and rice yield showed a strong positive correlation with soil Se concentrations, including total Se and bioavailable Se ($p < 0.001$, **Figure 5**). Selenite clearly promoted rice tillering and increased grain yield. Similarly, previous studies have reported that Se supplementation promotes rice growth (Moulick et al., 2016, 2018; Guan et al., 2018). Wang et al. (2013b) reported that rice treated with 21 g Se ha^{-1} produced more tillers per plant, more grains per panicle, bigger grains, and higher yields. The tillering capacity of rice depends mainly on genetic variation and environmental factors (light, temperature, and nutrients). The mineral nutrient Se is beneficial for the formation of rice tillers (Mu et al., 2021). Tiller number controls the panicle number of rice and plays a major role in determining grain yield. Additionally, as a beneficial element for plants, Se is believed to improve the agronomic traits of plants by regulating the activity of photosynthesis and enzymatic antioxidants in plant defense (Feng et al., 2015; Duan et al., 2019).

Selenium is applied to soil worldwide as a feasible and cost-effective method to produce Se-rich crops (Mora et al., 2015). In Finland, the government encourages the use of inorganic Se fertilizer to improve crop nutrition (Alfthan et al., 2015). In China, Se-rich rice was produced by an accurate Se supply (Wu et al., 2015). In a previous study (Dai et al., 2019), Se concentrations of brown rice increased with the elevated soil Se concentration (0.5–20 mg kg^{-1}). Similarly, compared with CK, Se treatment (5–200 mg kg^{-1}) significantly increased the grain Se concentrations in this study (**Figure 2A**). Soil total Se and bioavailable Se showed positive correlations with rice grain Se concentration (**Figure 5**). These correlations might be meaningful for accurate rice Se fertilization, rice grain Se biofortification, and the management of Se-rich soil. It is noteworthy that the rice growth and grain Se accumulation were still promoted by the 200 mg kg^{-1} Se treatment, indicating the high tolerance and accumulation ability of Se by the rice cultivar

TABLE 3 | The relative abundance (%) of taxon in the nematode community.

Se treatments	Trophic taxa					cp-value taxa			
(mg kg^{-1})	Bacterivores	Algivores	Fungivores	Herbivores	Omnivores–predators	cp-1	cp-2	cp-3	cp-4
0 (CK)	11.49 ± 2.17^{a}	69.17 ± 10.42^{a}	0.34 ± 0.58^{a}	4.39 ± 4.45^{a}	14.62 ± 8.03^{a}	6.15 ± 5.75^{b}	5.61 ± 3.42^{a}	73.62 ± 6.06^{a}	14.62 ± 8.03^{a}
5	3.70 ± 6.42^{a}	54.34 ± 42.64^{ab}	0.00 ± 0.00^{a}	37.64 ± 38.16^{a}	4.31 ± 5.96^{a}	3.70 ± 6.42^{b}	0.00 ± 0.00^{a}	91.99 ± 5.37^{a}	4.31 ± 5.96^{a}
10	42.59 ± 13.09^{a}	53.70 ± 18.33^{ab}	0.00 ± 0.00^{a}	3.70 ± 5.24^{a}	0.00 ± 0.00^{a}	41.2 ± 15.06^{ab}	0.00 ± 0.00^{a}	58.80 ± 15.06^{a}	0.00 ± 0.00^{a}
15	66.67 ± 16.67^{a}	22.22 ± 25.46^{ab}	0.00 ± 0.00^{a}	11.11 ± 19.25^{a}	0.00 ± 0.00^{a}	66.67 ± 16.67^{a}	0.00 ± 0.00^{a}	33.33 ± 16.67^{a}	0.00 ± 0.00^{a}
20	13.33 ± 11.55^{a}	46.67 ± 50.33^{ab}	0.00 ± 0.00^{a}	40.00 ± 40.00^{a}	0.00 ± 0.00^{a}	6.67 ± 11.55^{b}	6.67 ± 11.55^{a}	86.67 ± 11.55^{a}	0.00 ± 0.00^{a}
25	45.94 ± 20.83^{a}	2.56 ± 4.44^{b}	0.00 ± 0.00^{a}	51.50 ± 21.34^{a}	0.00 ± 0.00^{a}	37.61 ± 34.15^{ab}	8.33 ± 14.43^{a}	54.06 ± 20.83^{a}	0.00 ± 0.00^{a}
30	60.0 ± 52.92^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	40.00 ± 52.92^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	60.00 ± 52.92^{a}	40.00 ± 52.92^{a}	0.00 ± 0.00^{a}
35	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	100.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	21.43 ± 25.75^{a}	78.57 ± 25.75^{a}	0.00 ± 0.00^{a}
40	47.44 ± 46.21^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	52.56 ± 46.21^{a}	0.00 ± 0.00^{a}	47.44 ± 46.21^{ab}	19.23 ± 26.92^{a}	33.33 ± 57.74^{a}	0.00 ± 0.00^{a}
45	22.73 ± 32.14^{a}	0.00 ± 0.00^{b}	4.55 ± 6.43^{a}	72.73 ± 38.57^{a}	0.00 ± 0.00^{a}	4.55 ± 6.43^{b}	22.73 ± 32.14^{a}	72.73 ± 38.57^{a}	0.00 ± 0.00^{a}
50	25.00 ± 43.30^{a}	8.33 ± 7.22^{ab}	5.56 ± 9.62^{a}	56.94 ± 38.71^{a}	4.17 ± 7.22^{a}	0.00 ± 0.00^{b}	25.00 ± 43.3^{a}	65.28 ± 34.94^{a}	9.72 ± 8.67^{a}
100	5.56 ± 9.62^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	86.11 ± 12.73^{a}	8.33 ± 14.43^{a}	0.00 ± 0.00^{b}	22.22 ± 25.46^{a}	69.44 ± 17.35^{a}	8.33 ± 14.43^{a}
200	0.00 ± 0.00^{a}	7.41 ± 12.83^{ab}	7.41 ± 12.83^{a}	81.48 ± 32.08^{a}	3.70 ± 6.42^{a}	0.00 ± 0.00^{b}	44.44 ± 48.43^{a}	48.15 ± 44.91^{a}	7.41 ± 12.83^{a}

Different letters indicate a significant difference in the same column at the $p < 0.05$ level. Values represent mean ± SD.

Baixiang139. Even without Se supply (soil Se concentration of 0.42 ± 0.06 mg kg^{-1}), the Se concentration in rice grain reached 0.31 ± 0.22 mg kg^{-1} and beyond the Se-rich rice standard ranging from 0.04 to 0.30 mg kg^{-1} in China (Rich selenium paddy, GB/T 22499-2008). Therefore, the cultivar Baixiang139 could be used to produce Se-rich rice in the future, especially in Se-deficient areas.

The Effects of Selenite on Soil Nematode

Despite the occurrence of some harmful soil nematodes, overall, nematodes involved in nutrient cycling and energy flow contribute positively to ecosystem processes (Gebremikael, 2016). High nematode abundance has been demonstrated to be a symptom of a healthy soil ecosystem with the general presumption that "the more the better" (Yeates, 2003). The decrease of nematode abundance induced by selenite (**Figure 2B**) and a negative correlation between nematode abundance and soil bioavailable Se ($p < 0.01$) (**Figure 5**) were observed. Similarly, high concentrations of trace elements in agricultural soils, such as As, Zn, Cu, and Ni, have been reported to decrease nematode abundance in previous studies (Korthals et al., 1996; Park et al., 2011). The decreased nematode abundance may be achieved through two mechanisms. On the one hand, excessive Se disturbs protein expression and the antioxidant defense system directly in nematodes (Lv et al., 2021). On the other hand, it is likely that the changes in biotic (microorganisms and plant roots) and abiotic (soil properties) factors induced by Se decreased nematode abundance indirectly (Liu et al., 2016).

Besides nematode abundance, the nematode composition is also a focus of this study. A negative correlation between soil Se (total Se and bioavailable Se) with the relative abundance of algivores and bacterivores, and positive correlations between soil Se with the relative abundance of fungivores and herbivores were found (**Figure 5**). Algivirous nematodes are common in paddy soil (Okada et al., 2011). In this study, the main algivorous nematode, *Tobrilus*, showed a highly sensitive response to Se (**Figures 4**, **5**). Zhao and Neher (2013) conducted a methodical multivariate analysis and then pointed out that nematode genera (*Discolaimium*, *Discolaimus*, *Eudorylaimus*, etc.) correlated negatively with the soil Se shows potential in reflecting Se disturbance. Therefore, the sensitive *Tobrilus* can also be used in monitoring environmental Se disturbance in future studies. Herbivores feeding on plant root tissues or root exudates directly or indirectly affect the formation of nodules and mycorrhizae in plants and subsequently downregulate nitrogen fixation and other related functions. According to our results, the rise in herbivorous nematodes may be attributed to increased plant growth induced by Se supplementation. The increase of bacterivores and decrease of fungivores in the soil nematode community may inhibit soil mineralization compared with that in CK since they play a key role in nitrogen mineralization (Ferris et al., 1998). Considering the vital role nematodes play in soil health (Paz-Ferreiro and Fu, 2016), both the changes in abundance and composition of soil nematodes after a high Se supply indicate decreased soil biodiversity and function.

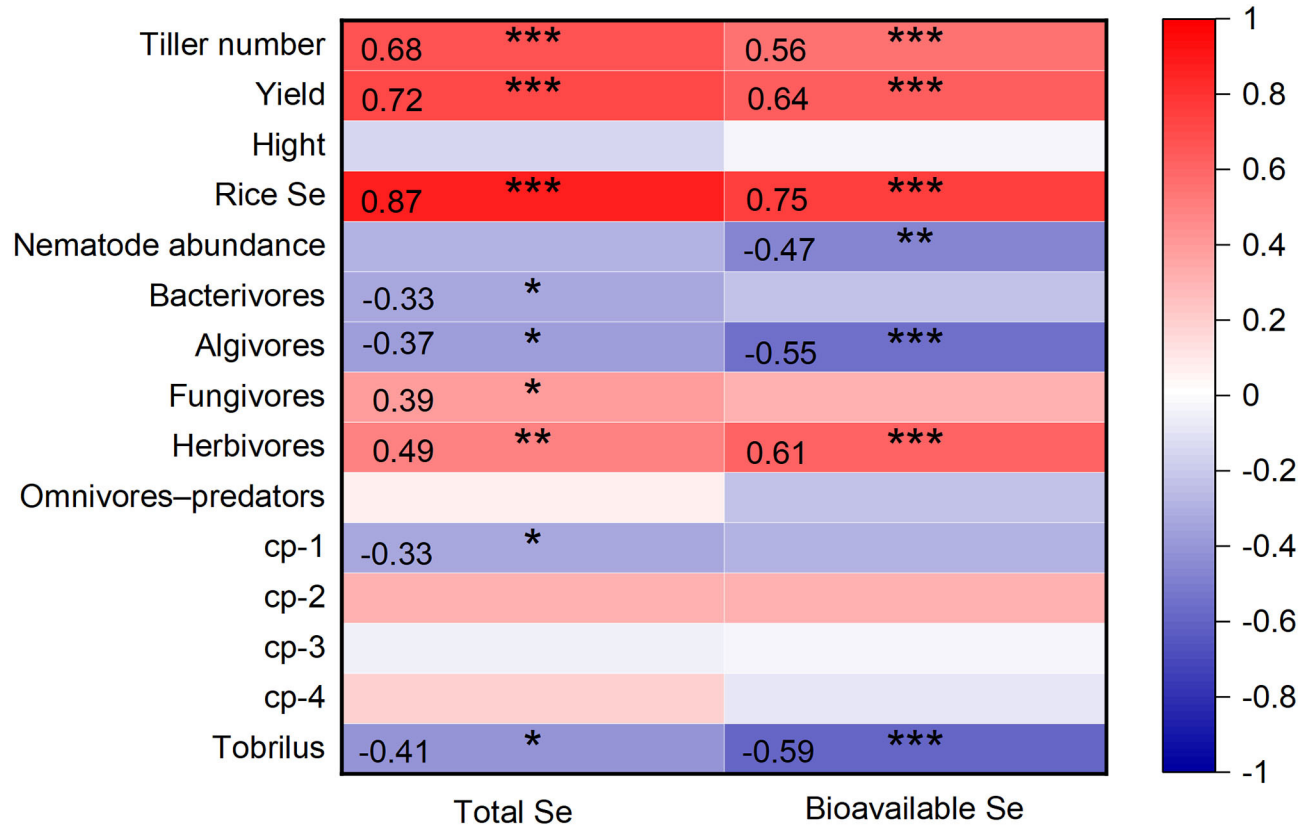

FIGURE 5 | Pearson correlations between the soil Se concentrations (total Se and bioavailable Se) with indexes of rice plant and soil nematodes ($n = 39$). *, **, and *** indicate significant relationships at $p < 0.05$, $p < 0.01$, and $p < 0.001$ level, respectively.

Balance Between Soil Se Biofortification of Rice With Soil Nematode Community Protection

On account of the low utilization of applied Se by crops, excessive Se might accumulate in soil and do harm to nearby ecosystems (Winkel et al., 2015). The soil fauna, for example, earthworms (Xiao et al., 2018) and collembola (Kuperman et al., 2018), have been reported to be reduced in the soil after Se exposure. The negative effects of excessive Se supply on soil nematodes were also proved by this study. To balance Se biofortification of rice with soil nematode community protection, a soil Se concentration threshold based on nematode response to Se supply is proposed here.

Based on the effect of Se on nematode abundance, the NOEC for soil total Se and bioavailable Se to nematodes was 9.25 and 1.88 mg kg^{-1}, respectively (**Figure 2B**). Based on the effect of Se on nematode genus composition, the NOEC for soil total Se and bioavailable Se was 7.90 and 1.33 mg kg^{-1}, respectively (**Figure 4**). Somogyi et al. (2006) collected soil samples from sunflower fields exposed to artificial selenite pollution for 7 years and analyzed the nematode community. The results demonstrated that the NOEC for soil total Se and bioavailable Se to nematode indexes (abundance, richness, etc.) is 7.25 and 2.09 mg kg^{-1}, which is consistent with the findings in this study. Additionally, the EC20 for total Se and bioavailable Se to nematode abundance were 1.45 and 0.21 mg kg^{-1}, respectively (**Figure 3**). Therefore, the lower values (total Se: 1.45 mg kg^{-1} and bioavailable Se: 0.21 mg kg^{-1}) were proposed to be soil Se thresholds to keep the nematode from Se disturbance according to the determining of ecological soil screening levels (US Environmental Protection Agency, 2005). At concentrations below soil Se thresholds, plant growth and Se accumulation in the grain will still be promoted, but the disturbance of the nematode community will be negligible. Therefore, the soil Se background concentration should be determined, and the amount of applied fertilizer should be strictly controlled to ensure a low soil Se level after Se fertilization.

Additionally, the method of Se biofortification with inorganic fertilizer can be replaced by approaches that are more friendly to nematodes, like organic Se fertilizers (Se-rich straw and animal manures) (Sharma et al., 2011; Bhatia et al., 2014). Organic fertilizers have been shown to promote soil nematode abundance in studies conducted in grassland and tillage fields (Benkovic-Lacic et al., 2013; Ikoyi et al., 2020). Foliar Se application could be used rather than soil Se application with greater efficiency of Se accumulation in maize (Wang et al., 2013a), wheat (Ros et al., 2016), and soybean (Yang et al., 2003). With ecological safety and economic feasibility (Yang et al., 2021), microbial fortification is

also considered to be a nematode-friendly method to produce Se-rich crops.

CONCLUSION

Overall, this study demonstrated that soil selenite supply (5–200 mg kg^{-1}) promoted plant growth and grain Se accumulation. However, the genus composition of nematodes changed significantly when 10 mg kg^{-1} Se or more were supplied. The abundance of nematodes decreased significantly when 15 mg kg^{-1} Se or more were supplied. These results indicate a potential risk of Se biofortification on soil nematodes. To balance Se biofortification of rice with soil nematode community protection, we suggest that the soil total Se concentration and bioavailable Se concentration after fertilization should be kept below 1.45 and 0.21 mg kg^{-1}, respectively. The effects of Se on nematode communities in different agricultural soils growing different crops should be analyzed in future investigations, together with the effects of added Se on soil physicochemical properties.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

XY and LY designed the framework of this study. JS performed the experiments and wrote the manuscript. XY, LY, XL, ZW, ZZ, ZQL, and QC revised this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.889459/full#supplementary-material

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Uptake and translocation mechanisms of different forms of organic selenium in rice (*Oryza sativa* L.)

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Selenium (Se) is an essential trace element for human and animal health, and toward an understanding of the uptake and translocation of Se in plants is important from the perspective of Se biofortification. In this study, we conducted hydroponic experiments to investigate the mechanisms of organic Se [selenomethionine (SeMet) and selenomethionine-oxide (SeOMet)] uptake, translocation, and the interactions between SeMet and SeOMet in rice. We also investigated differences in the dynamics of organic and inorganic Se uptake by rice roots. Concentration-dependent kinetic results revealed that SeMet uptake during a 1 h exposure was 3.19–16.0 times higher than that of three other Se chemical forms, with uptake capacity (V_{max}) values ordered as follows: SeMet>SeOMet>selenite>selenate. Furthermore, time-dependent kinetic analysis revealed that SeMet uptake by roots and content in shoots were initially clearly higher than those of SeOMet, although the differences gradually diminished with prolonged exposure time; while no significant difference was found in the transfer factor of Se from rice roots to shoots between SeMet and SeOMet. Root uptake of SeOMet was significantly inhibited by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (30.4%), $AgNO_3$ (41.8%), and tetraethylammonium chloride (TEACl) (45.6%), indicating that SeOMet uptake is a metabolically active process, and that it could be mediated *via* aquaporins and K^+ channels. Contrarily, SeMet uptake was insensitive to CCCP, although markedly inhibited by $AgNO_3$ (93.1%), indicating that rice absorbs SeMet primarily *via* aquaporins. Furthermore, Se uptake and translocation in rice treated simultaneously with both SeMet and SeOMet were considerably lower than those in rice treated with SeMet treatment alone and notably lower than the theoretical quantity, indicating

interactions between SeMet and SeOMet. Our findings provide important insights into the mechanisms underlying the uptake and translocation of organic Se within plants.

KEYWORDS

rice, selenomethionine, selenomethionine-oxide, uptake kinetics, transport, interaction

Introduction

Selenium (Se), an essential micronutrient with respect to human and animal health, is a necessary component of more than 30 Se-containing proteins and enzymes in mammals and is associated with multiple properties, including antioxidative, immunological, and anticarcinogenic effects (Rayman, 2012; Avery and Hoffmann, 2018). Indeed, a deficiency in Se can lead to the risk of cardiovascular and cancer diseases (Hatfield et al., 2014). Although the World Health Organization (WHO) recommends a daily Se intake of 50–200 $\mu g\ d^{-1}$ for adults, given marked differences in the soil contents of Se worldwide and the distribution of Se-poor soils in some notably populous regions, Se deficiency may afflict as many as one billion people globally (Combs, 2001; Haug et al., 2007).

Selenium has multiple beneficial effects on the plant growth at appropriate concentration, including the enhancement of antioxidant capacity and photosynthesis (Jiang et al., 2021; Lanza et al., 2021a); while excessive Se can be detrimental (Lanza et al., 2021b). Concerning its beneficial effects on plant growth and human health, plants can be strategically utilized to regulate the effects of Se on both. Consumption of Se-rich plant food is considered to be the most effective approach to increase human Se uptake (White and Broadley, 2009; Alfthan et al., 2015), and accordingly, in those regions characterized by Se-deficient soils, agronomic biofortification based on Se fertilization could be practiced to produce Se-rich crops, thereby enhancing human Se intake.

Among different factors influencing the accumulation of Se in plants, the uptake and translocation of this element are the most fundamental physiological aspects. Consequently, gaining an in-depth understanding of the associated processes and mechanisms is important from the perspective of developing Se biofortification strategies. In natural environment, a range of distinct chemical forms of Se exists, and among which, selenite (SeIV) and selenate (SeVI) are the two most abundant forms in soil, with selenite predominating in soils characterized by intermediate redox potentials and selenate predominating under aerobic and neutral to alkaline conditions (Elrashidi et al., 1987). Although both selenate and selenite can be absorbed from soil by plant roots, it appears that neither is taken up *via* Se-specific transporters. Selenate is typically taken up *via* sulfate transporters (Sors et al., 2005; Mehdawi et al., 2018), and selenite might be absorbed *via* silicon influx transporters (Zhao et al., 2010) or incorporated into an active process mediated by phosphate transporters (Li et al., 2008; Zhang et al., 2014). Upon uptake, most selenite is rapidly metabolized to organic Se compounds and retained within the root system, whereas selenate can be rapidly translocated to the shoots (Li et al., 2008; Huang et al., 2017; Gong et al., 2018; Yu et al., 2019). In some soils, Se is also present as organic forms, such as SeMet and SeCys (Kikkert and Berkelaar, 2013); however, compared with those of the inorganic forms of Se, the uptake mechanisms of organic forms of Se is less investigated.

Rice (*Oryza sativa* L.) is a staple food for nearly half of the world's population and one of major sources of the dietary Se intake (Rayman et al., 2008). In this regard, in addition to the total concentration of Se in crops, Se speciation is of particular importance in terms of its different health benefits (Zhu et al., 2009). Research has shown that in the mature grains of rice, Se is present primarily in organic forms, and among which, selenomethionine (SeMet) predominates (Sun et al., 2010; Huang et al., 2018). SeMet can be incorporated into proteins either directly or non-specifically *via* the replacement of methionine and is thereby readily absorbed by humans (Fairweather-Tait et al., 2010). Moreover, the uptake rate of SeMet by plants is higher than that of either selenite or selenate (Kowalska et al., 2020; Wang M. K. et al., 2020). Selenomethionine-oxide (SeOMet) is a derivative obtained from the transformation of SeMet. In our previous study, we detected SeOMet in the soil solution extracted from natural and selenite-supplied soils (Li et al., 2010), and SeOMet was also found in plants growing in media supplemented with either selenite or SeMet (Li et al., 2008; Kowalska et al., 2020). However, the uptake of SeOMet in plant roots remain unclear, and differences between organic and inorganic Se uptake are insufficiently well documented. Since SeMet and SeOMet exist in the forms of uncharged molecules, we speculate that the uptake of these two Se forms might be mediated by aquaporins, with high uptake potential in plant roots. In addition, when supplied with different chemical forms of Se simultaneously, a non-additive effect on the uptake would occur, for example, the presence of selenite appeared to inhibit the uptake of selenate in plants (Li et al., 2008; Wang et al., 2019). Thus we speculate a certain interaction might occur between SeMet and SeOMet during uptake process. To gain further insights in these regards, we conducted a series of hydroponic experiments to investigate (1)

differences between the dynamics of organic and inorganic Se uptake by rice roots, (2) the mechanisms associated with the uptake of SeMet and SeOMet by roots and their subsequent translocation in plant, and (3) the interactions between SeMet and SeOMet during the uptake and translocation processes. We anticipated that the findings of this study would provide a theoretical basis for increasing Se levels and accumulating more organic Se in crops.

Materials and methods

Plant culture

For the purposes of this study, we used the rice (*Oryza sativa* L.) cultivar Zhuliangyou120 (a common *indica*-type cultivar). Rice seeds were surface sterilized with 30% (v/v) H_2O_2 for 15 min, rinsed thoroughly with deionized water, soaked in saturated $CaSO_4$ solution in the dark overnight, and then germinated in a 0.5 mM $CaCl_2$ solution. Seven days after germination, rice plants were transplanted into plastic pots containing 3 L of modified ½ Kimura nutrient solution (Huang et al., 2015), with the following composition (mmol L^{-1}): KNO_3 0.091, KH_2PO_4 0.1, Ca $(NO_3)_2{\cdot}4H_2O$ 0.183, $MgSO_4{\cdot}7H_2O$ 0.274, $(NH_4)_2SO_4$ 0.183, Fe (e1-EDTA 6.0×10^{-2}, $ZnSO_4{\cdot}7H_2O$ 1.0×10^{-3}, H_3BO_3 3.0×10^{-3}, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1.0×10^{-3}, $MnSO_4{\cdot}H_2O$ 1.0×10^{-3} and $CuSO_4{\cdot}5H_2O$ 2.0×10^{-4}. The pH of the solution was buffered with 2 mM 2-morpholinoethanesulfonic acid (MES) and adjusted to a value of 5.5 with either 1 mM KOH or HCl. The solution was renewed at 3-day intervals.

Hydroponic experiments were conducted on a bench within a greenhouse of the China Agricultural University, Beijing. The conditions of the growth environment was as follows: day/night temperatures of 30 ± 2°C/23 ± 2°C; a light period of 14 h, with illumination provided by natural sunlight supplemented with sodium vapor lamps to maintain a light intensity of 240–350 μmol m^{-2} s^{-1}; and a relative humidity of 60–70%.

Selenium sources

Selenite and selenate were obtained from Sigma-Aldrich (St Louis, MO, United States), SeMet was provided by Shanxi University, and SeOMet was prepared by reacting SeMet with 3% H_2O_2 under sonication for 1 h (Larsen et al., 2004).

Concentration-dependent kinetics of Se uptake

To evaluate the uptake capacities of rice roots with respect to organic and inorganic Se, we transferred 4-week-old rice plants to 250 mL of Se uptake solutions, each containing different chemical forms of Se, namely, selenite, selenate, SeMet, and SeOMet. For each of the different uptake solutions, a series of Se treatment solution were prepared with the Se concentration ranging from 0 to 20 μM (0, 0.1, 0.5, 1, 5, 10, and 20 μM at pH 5.5). Each treatment had four replicates with one plant per replicate. After 1 h of uptake incubation, rice roots were rinsed three times with deionized water and then transferred to 150-mL of ice-cold desorption solution (1 mM $CaSO_4$ + 2 mM MES, pH 5.5) for 15 min to remove the Se adsorbed on root surfaces (Li et al., 2008). Following desorption, the rice roots were rinsed three times in deionized water and thereafter separated from the shoots. The root samples were oven-dried at 105°C for 30 min and 75n for 48 h, after which, they were weighed and used for determinations of Se concentrations.

Time-dependent kinetics of selenomethionine and selenomethionine-oxide uptake

This experiment was conducted to investigate the temporal patterns of organic Se uptake and translocation by rice. Four-week-old rice plants were transferred to 1-L plastic container (one plant per pot) containing uptake solutions [with normal nutrients (control) and supplemented with either 5 μM SeMet or 5 μM SeOMet (2 mM MES, pH 5.5)], to which they were exposed for 1, 3, 5, 18, 26, 48, or 72 h, a control treatment (without any Se) was also conducted. Each treatment had four replicates. Following organic Se absorption, the roots were rinsed with deionized water and desorbed as described previously. The roots and shoots were then oven-dried and analyzed for Se concentrations.

Effects of inhibitors on the uptake of selenomethionine and selenomethionine-oxide

To investigate the physiological processes and mechanisms of organic Se uptake, we examined the effects of the following inhibitors on the uptake of Se by rice: $AgNO_3$, $CoCl_2$, tetraethylammonium chloride (TEACl), 4,4-diisothiocyanatostilbene-2,2-disulfonic acid disodium salt hydrate (DIDS), and carbonyl cyanide 3-chlorophenylhydrazone (CCCP). $AgNO_3$ is an aquaporin inhibitor that inhibits the water permeability of root cell plasma membranes (Niemietz and Tyerman, 2002), whereas $CoCl_2$, TEACl, and DIDS act as inhibitors of Ca^{2+} (Harada and Shimazaki, 2009), K^+ (White, 1995), and anion channels (Zhang et al., 2013), respectively, and the protonophore CCCP is a metabolic inhibitor. All inhibitors used in the study were obtained from Sigma-Aldrich (St Louis, MO, United States).

Four-week-old plants were transferred to uptake solutions containing 5 μM organic Se (SeMet or SeOMet) and different inhibitors (100 μM $AgNO_3$, 5 mM TEACl, 5 mM $CoCl_2$, 100 μM DIDS, or 1 μM CCCP, respectively), a control treatment (without any Se) was also conducted. CCCP was dissolved in ethanol and added to the solution at a final concentration of 0.01% (v/v) (Li et al., 2008). Consequently, we also included an additional control treatment containing 0.01% (v/v) ethanol. Four replicates were used for each treatment. After exposure for 1 h, the treated roots were rinsed with deionized water and the Se adsorbed on root surfaces was desorbed as described previously. Thereafter, the roots were oven-dried and analyzed for Se concentrations.

Effects of P or S starvation on selenomethionine and selenomethionine-oxide uptake and translocation

This experiment was conducted to investigate whether phosphorus (P) or sulfur (S) starvation would influence the uptake and translocation of organic Se by rice. Four-week-old plants were transferred to 1-L plastic containers and treated with normal, P-deficient, or S-deficient nutrient solutions for 7 days. In the P-deficient and S-deficient solution, $MgSO_4$, $ZnSO_4$, KH_2PO_4, or $CuSO_4$ were replaced by the corresponding chloride salts. At the end of the treatment period, the plants were transferred to a normal nutrient solution (modified ½ Kimura nutrient solution), to which either 5 μM SeMet or 5 μM SeOMet was added, followed by incubation for a further 2 days, a control treatment (without any Se) was also conducted. Then, the roots were desorbed, rinsed, oven-dried, and analyzed for Se concentrations. Each treatment had four replicates.

Interactions between selenomethionine and selenomethionine-oxide

In this experiment, we sought to characterize the interactions between SeMet and SeOMet during their uptake and translocation in rice plants. Four-week-old plants were transferred to 250-mL containers, containing one of three different absorption solutions with the same total Se concentration (5 μM SeMet, 5 μM SeOMet, or 2.5 μM of both SeMet and SeOMet), a control treatment (without any Se) was also conducted. Each treatment had four replicates. After exposure for 1 h, the rice roots were rinsed three times with deionized water and desorbed as described previously. Thereafter, the roots and shoots were harvested, washed, oven-dried, and analyzed for Se concentrations.

Analysis of Se content

For Se content analyses, 0.2500 g dried root and shoot samples were digested with 8 mL HNO_3 (Guaranteed reagent) using a CEM MARS5 microwave sample preparation system (CEM Corp., Matthews, NC, United States). A 4 mL volume of the digest solution was then mixed with 1 mL of 6 M HCl and heated at 95 for 2 h to reduce selenate to selenite. Concentrations of Se in the mixed solution were determined by atomic fluorescence spectrometry using an AFS-920 Dual-channel Atomic Fluorescence Spectrometer, (Beijing Jitian Instruments Co., Ltd., Beijing, China). For quality assurance, we simultaneously analyzed a certified reference material (GBW10014, cabbage) and blanks, the recovery of Se in GBW10014 was 85–110%.

Data analysis

Se uptake kinetics were described based on the Michaelis–Menten Equation:

$$V = \frac{V_{\max} \times C}{K_{\mathrm{m}} + C}$$

where V represents the uptake rate [μg g^{-1} root DW (dry weight) h^{-1}], K_m represents the Michaelis constant (μM), V_{max} represents the maximal uptake rate (μg g^{-1} root DW h^{-1}), and C represents the substrate concentration (μM). The Michaelis–Menten equation is of particular value with respect to the evaluation of transporter-mediated uptake processes. Uptake capacity (V_{max}) is the maximal transport rate when all available carrier sites are saturated, whereas substrate affinity (K_m) is equal to the substrate concentration at which the reaction rate is half-maximal.

All results are expressed as mean values with corresponding standard errors ($n = 4$). Two-way analysis of variance (ANOVA) was performed to test the significance of the Se form, treatment time (or inhibitor type, nutrient status) and the interactions between them, by using SAS 9.3 statistical software with least significant difference (LSD, $P < 0.05$).

Results

Concentration-dependent kinetics of Se uptake

We found that uptake of the four assessed Se chemical forms by rice roots increased concomitant with an increase in the Se concentration of the uptake solution, all of which were satisfactorily described by the Michaelis–Menten equation (**Figure 1** and **Table 1**). With the exception of SeMet, the influx of the remaining three chemical forms of Se into roots

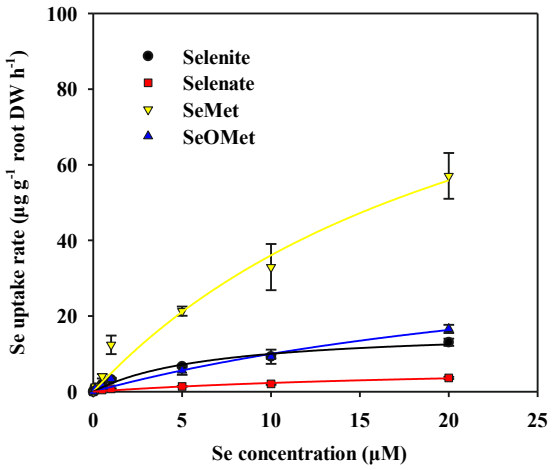

FIGURE 1
Concentration-dependent kinetics for the influx of different forms of Se into rice roots within 1 h. Data are presented as mean ± SE (n = 4). The curves represent the fitted Michaelis–Menten kinetics.

had features of saturating kinetics within the concentration range from 0 to 20 μM (Figure 1). The Michaelis–Menten kinetics curves also showed that, in the uptake solution containing 5–20 μM Se, the rate of SeMet uptake was 3.19–16.0 times higher than that of the other three Se chemical forms. In addition, we established that the chemical forms of Se had a marked effect ($P < 0.05$) on V_{max} and K_m values (Table 1). The calculated values of V_{max} declined in the order of SeMet > SeOMet > selenite > selenate, thereby indicating that organic forms of Se, particularly SeMet, are characterized by a considerably higher uptake potential in rice roots than inorganic forms. Moreover, we found that the K_m values calculated for selenate, SeMet, and SeOMet uptake were 2. 42-, 2. 64-, and 4.18-fold higher than those of selenite, respectively, indicating that rice roots have a higher affinity for selenite than for the other assessed Se chemical forms. Interestingly, we observed that the absorption kinetics of selenite and SeOMet in rice differed according to exogenous Se concentrations. Specifically, at Se concentrations between 0 and 10 μM, the rate of selenite uptake was higher than that of SeOMet ($P > 0.05$), whereas the opposite response (selenite < SeOMet) was detected at higher Se concentrations ranging from 10 to 20 μM ($P > 0.05$), and the two corresponding Michaelis–Menten curves had a single point of intersection at 10 μM (Figure 1).

Time-dependent kinetics of organic Se uptake and translocation

Our time-dependent analysis of organic Se uptake revealed it was significantly affected by Se form, treatment time, and their interaction between these two factors ($P < 0.001$). And the Se in rice plants in the control treatment was below the detection level (the same below). Generally, the uptake rate of Se as SeMet was higher than that of the SeOMet form at all assessed time points, although the differences narrowed with time. For example, although SeMet uptake rate after exposure for up to 26 h was 1.56–7.19-fold higher than that of SeOMet, however, after exposure for 48 h, the uptake rate of Se as SeMet was only 9.60–32.8% higher (Figure 2A). Furthermore, irrespective of the chemical forms, the uptake rate of Se increased with a prolongation of treatment time, although the increase in SeMet uptake rate declined with time and that of SeOMet remained relatively constant. In neither case, however, did the rate of Se uptake reach a plateau during the assessed treatment period. A similar tendency was obtained with respect to the total Se uptake: within 26 h, the total Se in SeMet treatments was 1.89–7.09-fold higher than that of SeOMet treatments; while the difference was only 8.82–19.8% after 48 h (Figure 2B).

When assessed at time points prior to 26 h, the contents of Se in the shoots of plants treated with SeOMet were between 58.6 and 72.3% lower than those in the plants treated with SeMet. However, in response to prolonged exposure for 48 and 72 h, the differences between these two Se chemical forms became non-significant (Figure 2C). In order to evaluate the root-to-shoot translocation ability of different Se sources, the transfer factor was introduced in the study. In general, irrespective of the Se treatments, the transfer factor of Se from rice roots to shoots showed an increasing trend with increase of the exposure time, varied from 0.025 to 0.302; while the lowest transfer factor was appeared at 18 h. In addition, there was no significant difference of the transfer factor between SeMet and SeOMet treatments at all exposure time (Figure 2D).

TABLE 1 Kinetic parameters for the influx of four forms of Se into rice roots.

Treatment	V_{max} (μg·g^{-1} root DW h^{-1})	K_m (μM)	R
Selenite	16.9 ± 2.16	6.67 ± 2.20	0.988***
Selenate	7.67 ± 3.24	22.8 ± 15.7	0.973**
SeMet	125 ± 42.2	24.3 ± 13.0	0.986***
SeOMet	45.1 ± 20.7	34.6 ± 22.9	0.985***

***$p < 0.001$; **$P < 0.01$.

Effects of inhibitors on the uptake rate of organic Se

To gain further insights into the uptake process, we examined the effects of exposure to four specific inhibitors on uptake of the two assessed chemical forms of organic Se following 1 h exposures. Our observations revealed that Se uptake rate was significantly affected by Se form, inhibitor

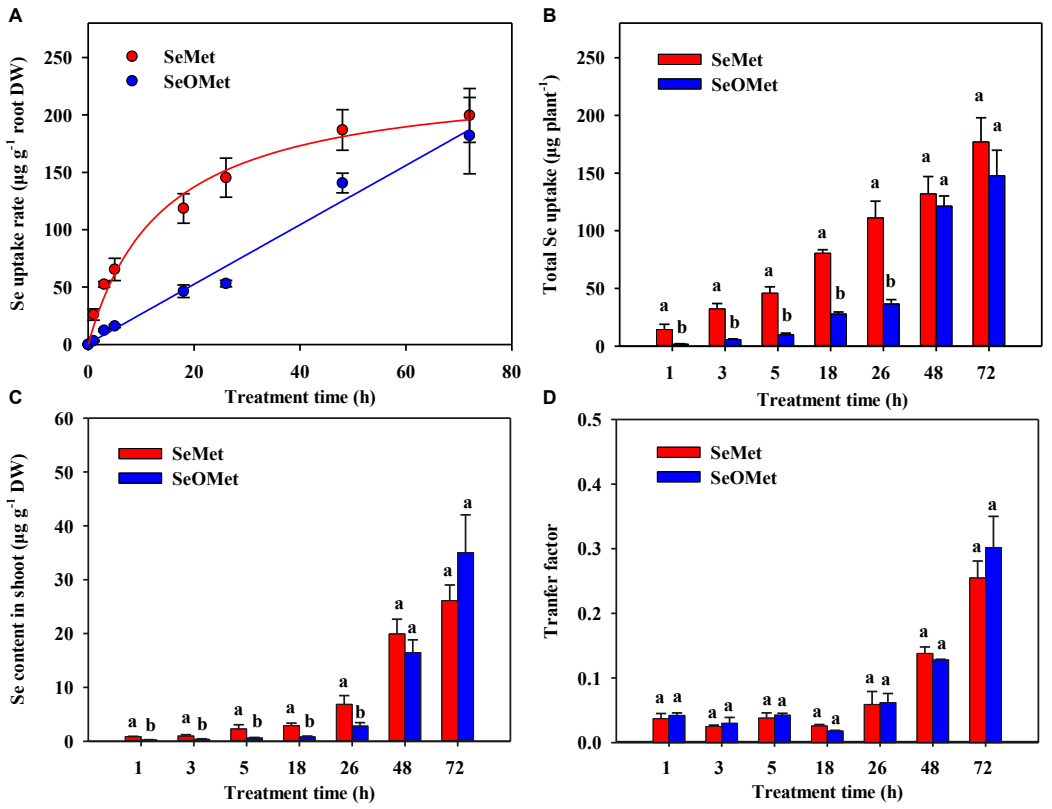

FIGURE 2
Time-dependent kinetics of the uptake of organic Se by rice roots **(A)**, total Se uptake **(B)**, the contents of Se in rice shoots **(C)**, and the transfer factor of Se from roots to shoots **(D)**. Data are presented as mean ± SE (n = 4). Different lowercase letters above bars indicate significant differences between SeMet and SeOMet treatments in individual treatment times ($P < 0.05$).

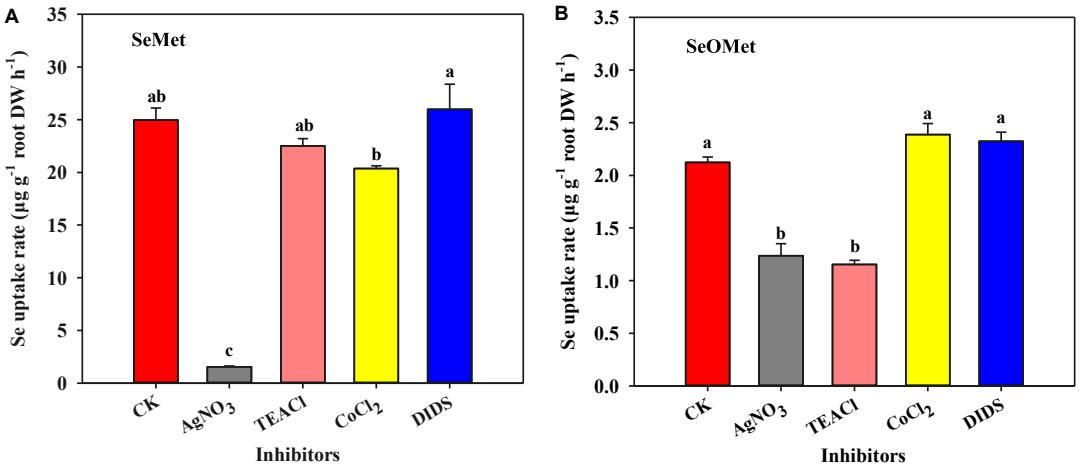

FIGURE 3
Effect of different specific inhibitors on the uptake of Se by rice supplied with SeMet **(A)** and SeOMet **(B)**. Data are presented as mean + SE (n = 4). Different lowercase letters above bars indicate significant differences among the inhibitor treatments ($P < 0.05$).

type, and their interaction between these two factors (Figure 3, $P < 0.001$). Generally, the uptake of Se as SeMet by rice roots was substantially higher than that as SeOMet, the former being 1.25–18.5-fold higher than the latter. Compared with the control, the exposure to $AgNO_3$ significantly reduced the uptake of Se as SeMet and SeOMet by 93.1 and 41.8%,

respectively ($P < 0.05$); whereas, although TEACl significantly inhibited the uptake of Se as SeOMet by 45.7% ($P < 0.05$), no significant inhibitory effect was detected in the uptake of SeMet. Contrastingly, neither $CoCl_2$ nor DIDS had any significant inhibitory effects on Se uptake as either SeMet or SeOMet.

Moreover, whereas the exposure to the metabolic inhibitor CCCP had no significant effect on the uptake of Se as SeMet, a significant reduction of 30.4% was detected for SeOMet form. The presence of ethanol in this treatment had no significant effect on Se uptake (**Figure 4**).

Effects of P or S starvation on organic Se uptake and translocation

This experiment was designed to determine the effects of major macronutrients P and S on the uptake and translocation of different organic chemical forms of Se. Rice plants were grown in normal, S-deficient, or P-deficient medium for 7 days, after which, they were transferred to media containing either 5 μM SeMet or SeOMet for a further 2 days. To evaluate the efficiency of Se translocation, Se distribution in shoot (%) was measured as the proportion of Se allocated to rice shoots. Interestingly, we found that whereas the uptake of Se by roots was significantly affected by Se forms ($P < 0.05$) but not by S or P status ($P > 0.05$), Se distribution in shoot (%) was significantly affected by nutrient status ($P < 0.05$) but not by Se forms ($P > 0.05$). Moreover, the contents of Se in shoots were significantly affected by both Se forms ($P < 0.05$) and the nutrient status ($P < 0.05$) (**Table 2**).

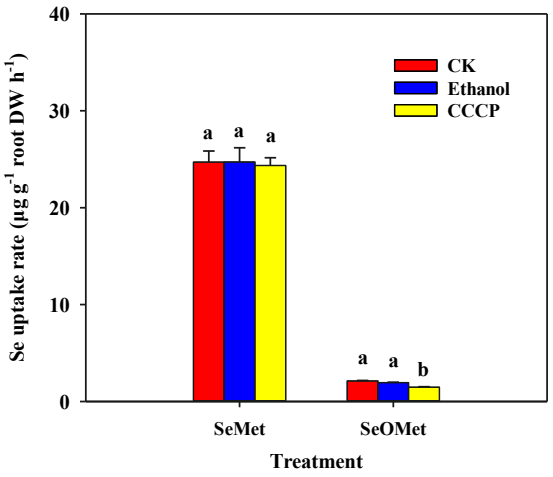

FIGURE 4
Effect of the respiratory inhibitor CCCP on the uptake of Se by rice supplied with different forms of organic Se. Data are presented as mean + SE ($n = 4$). Different lowercase letters above bars indicate significant differences among inhibitor treatments in individual Se treatment forms ($P < 0.05$).

In general, the contents of Se detected in rice plants, Se uptake rate, total uptake of Se, and distribution in shoots were higher in plants administered SeMet (although not all the detected differences were significant). Furthermore, compared with the normal treatment, neither P nor S deficiencies appeared to have any substantial effects on either the uptake or translocation of Se by rice, although we did observe a non-significant 14.9% reduction in the uptake of Se as SeOMet in response to a deficiency in P (**Table 2**).

Interactions between selenomethionine and selenomethionine-oxide

The results of interaction experiment revealed that Se uptake and translocation in rice were significantly affected by the interactions between SeMet and SeOMet (**Table 3**). We found that both root Se content and uptake in rice treated with both SeMet and SeOMet forms at the same total Se concentration (SeMet + SeOMet) were 90.9 and 92.5%, respectively, lower than those in plants exposed to SeMet only, although no significant differences were detected between SeMet + SeOMet and SeOMet treatments.

The efficiency of Se translocation during 1 h treatments was also expressed in terms of Se distribution in shoot (%). However, we were able to detect Se only in the shoots of those plants treated with SeMet treatment, which accounted for 16.9% of the total Se. In addition, the total uptake of Se by the roots of plants exposed to SeMet was between 11.4- and 11.7-fold higher than that in the SeOMet and SeMet+SeOMet treatments ($P < 0.001$). According to the calculations described by Longchamp et al. (2013) and Wang et al. (2019), in the absence of an interaction between two Se chemical forms, the total uptake of Se by roots should theoretically be 5.01 μg in the SeMet + SeOMet treatment. However, our findings indicated a total Se uptake of only ~15% of the theoretical quantity in plants exposed to a mixture of SeMet and SeOMet, which accordingly tends to indicate a non-additive effect. This suggests that during uptake and translocation in rice, SeMet and SeOMet may interact to a certain extent.

Data are presented as the mean ± SE ($n = 4$). Different letters after values within the same column indicate a significant difference among the treatments ($P < 0.05$).

Discussion

Selenium uptake kinetics in rice root

The capacity of plants to take up Se from soil depends on plant species, soil Se concentration and form, and environmental conditions (including pH, Eh, and organic

matter content). A number of previous studies have reported the uptake kinetics of different Se chemical forms in plant roots. For example, Huang et al. (2015) observed that the uptake of selenite into rice roots was considerably more rapid than that of selenate, with the value of V_{max} for selenite influx (102 $\mu g \cdot g^{-1}$ root h^{-1} DW) being approximately 6.5-fold higher than that for selenate (13.7 $\mu g \cdot g^{-1}$ root h^{-1} DW), with K_m values of 16.2 and 11.3 μM for selenite and selenate, respectively. Similarly, Hu et al. (2018) demonstrated that, for Se uptake by wheat roots, selenite (V_{max}: 25.6 $\mu g \cdot g^{-1}$ root h^{-1} DW) is characterized by a higher uptake potential than nanoselenium (V_{max}: 10.1 $\mu g \cdot g^{-1}$ root h^{-1} DW). Moreover, Zhang et al. (2019) obtained a V_{max} value of 132 $\mu g \cdot g^{-1}$ root h^{-1} DW for the uptake of SeMet. In the present study, we found that rice are characterized by a higher uptake potential (V_{max}) for organic Se than for inorganic forms, showing a descending order of SeMet > SeOMet > selenite > selenate (**Figure 1** and **Table 1**). Similarly, Wang M. K. et al. (2020) demonstrated that the roots of maize (*Zea mays* L.) had a higher uptake of organic Se (SeMet, MeSeCys, and SeCys) than inorganic Se (selenite and selenate) when supplied with 0.01 or 1 $mg \cdot L^{-1}$ Se. Consistently, Kikkert and Berkelaar (2013) found that the rate of SeMet uptake by the wheat roots was considerably higher than that of either selenite or selenate and conjectured that this difference could be attributable to the differences in the activities of their respective transporters. Moreover, in the present study, the differences in the rates of selenite and SeOMet uptake by rice roots varied according to the Se concentration exogenously applied Se (**Figure 1**). Likewise, Kikkert and Berkelaar (2013) found that the rate of selenite uptake was 60% lower than that of selenate, when supplied at a Se concentration of 0.5 μM but was 3.6 times higher when supplied with 5 μM Se. Collectively, these findings tend to indicate that the Se uptake capacity of plants might also be influenced by the interaction between the Se chemical form and the Se exposure level.

Regarding the two organic Se compounds (SeMet and SeOMet), this study showed that the uptake rate of SeMet was significantly greater than that of SeOMet during the initial 26 h of treatments (**Figures 1**, **2**), although the extent of the observed differences narrowed when measured at 48 and 72 h (**Figure 2**). We speculated that this pattern reflected the fact that during the latter part of the treatment period, SeMet uptake was gradually approaching a level of saturation at a decreasing rate. Additionally, the effects of exposure time on organic Se absorption could be attributed to the transformation of SeMet to SeOMet in rice roots or within the rhizosphere, a conjecture that is supported by previous findings indicating that SeOMet is detectable in the roots

TABLE 2 Effect of Se chemical forms supplied and nutrient status on the content, uptake rate, and proportion of Se allocated to rice shoot.

Treatment		Se content ($\mu g\ g^{-1}$ DW)		Se uptake rate	Shoot-Se%	Total Se uptake ($\mu g\ plant^{-1}$)
		Root	Shoot	($\mu g\ g^{-1}$ root DW)		
SeMet	Normal	97.4 ± 3.23a	20.2 ± 1.96a	140.7 ± 4.58a	30.6 ± 2.63a	190.0 ± 15.9a
	S-deficient	91.7 ± 3.87a	21.6 ± 1.51a	134.9 ± 7.54a	31.8 ± 1.00a	190.8 ± 14.2a
	P-deficient	100.6 ± 1.23a	18.4 ± 0.97a	138.2 ± 2.45a	27.2 ± 1.66a	199.4 ± 8.93a
SeOMet	Normal	95.4 ± 3.58a	16.8 ± 1.07ab	131.2 ± 4.04a	27.2 ± 1.79ab	181.5 ± 11.8a
	S-deficient	91.6 ± 4.54a	19.2 ± 1.17a	130.8 ± 5.95a	29.9 ± 1.46a	189.9 ± 15.9 a
	P-deficient	87.3 ± 1.47a	14.7 ± 0.70b	116.8 ± 2.92a	25.2 ± 0.87b	166.7 ± 6.85 a
Se treatment (A)		$P = 0.0688$	$P = 0.0076$	$P = 0.0093$	$P = 0.0936$	$P = 0.1480$
Nutrient status (B)		$P = 0.3650$	$P = 0.0262$	$P = 0.2481$	$P = 0.0378$	$P = 0.8120$
A × B		$P = 0.1150$	$P = 0.8583$	$P = 0.2224$	$P = 0.8782$	$P = 0.3650$

Data are presented as mean ± SE ($n = 4$). Different letters after values in the same column indicate significant differences among plants with different nutrient status ($P < 0.05$).

TABLE 3 Effect of SeMet and SeOMet interaction on the uptake and translocation of Se by rice.

Treatment	Se content ($\mu g\ g^{-1}$ DW)		Se uptake rate ($\mu g\ g^{-1}$ root DW h^{-1})	Shoot-Se (%)	Total Se uptake ($\mu g\ plant^{-1}$)
	Root	Shoot			
SeMet	22.98 ± 0.53a	1.87 ± 0.33	27.70 ± 1.00a	16.9 ± 1.6	9.30 ± 0.58a
SeOMet	1.93 ± 0.13b	ND	1.93 ± 0.13b	ND	0.73 ± 0.06b
SeMet+SeOMet	2.08 ± 0.08b	ND	2.08 ± 0.08b	ND	0.75 ± 0.05b
P	<0.001	–	<0.001	–	<0.001
Theoretical quantity	11.9 ± 1.05	0.93 ± 0.18	14.2 ± 1.50	15.7 ± 1.55	5.01 ± 0.58

The theoretical quantity calculated for the different proportions of SeMet and SeOMet treatments is based on the actual measured Se contents in rice tissues in single-SeMet or SeOMet treatments. Data are presented as the mean ± SE ($n = 4$). Different letters after values within the same column indicate a significant difference among the treatments ($P < 0.05$).

of lettuce (*Lactuca sativa* L.) exposed to SeMet (Kowalska et al., 2020). Similarly, in a previous study, we detected both SeMet and SeOMet in the roots of selenite-treated wheat (Li et al., 2008), thereby indicating an occurrence of the oxidative transformation of Se in plants.

The mechanisms of selenomethionine and selenomethionine-oxide uptake

To investigate the physiological processes associated with the uptake of SeMet and SeOMet by rice and the underlying mechanisms, effects of selected inhibitors on Se uptake have been assessed in this study. Among these, CCCP is a respiratory inhibitor that promotes a dissipation of the proton motive force across membranes. We found that whereas the uptake of SeOMet is sensitive to CCCP, but unaffected on SeMet (**Figure 3**). These observations contrast with the findings of previous hydroponic studies, which have revealed that CCCP significantly inhibits the uptake of SeMet by rice and wheat, thereby tending to indicate that SeMet is taken up by an energy-dependent symport process (Abrams et al., 1990; Zhang et al., 2019). We suspect that the disparity between the findings of these different studies could be due to differences in the respective exposure times (only 1 h in this study), which should be confirmed by further studies. Nevertheless, our findings do indicate that SeOMet uptake is a metabolically active process requiring selective binding sites and metabolic energy.

Furthermore, the findings of our specific inhibitor treatments indicate that SeMet and SeOMet are taken up into rice roots *via* different channels. Among the other inhibitors we studied, $AgNO_3$ is a potential inhibitor of aquaporins of plant origin and partially inhibit the uptake of selenite (Zhang et al., 2006) and Nano-Se (Wang M. K. et al., 2020). The mechanism of its inhibit function is that silver reacts with the sulfhydryl group of a cysteine and also with a histidine, thereby resulting in a gating of the targeted aquaporins (Niemietz and Tyerman, 2002). The other three assessed inhibitors, TEACl, $CoCl_2$, and DIDS, are recognized as specific inhibitors of K^+ (White, 1995), Ca^{2+} (Harada and Shimazaki, 2009), and anionic channels, respectively. In the present study, we found that the addition of $AgNO_3$ to the uptake solution significantly reduced the rate of SeMet uptake by 93.1% (**Figure 3**), thereby providing evidence that rice absorbs SeMet mainly *via* aquaporins. With respect to SeOMet, we observed that both $AgNO_3$ (41.8%) and TEACl (45.6%) can significantly inhibit uptake by rice roots (**Figure 3**), which might indicate that the influx of SeOMet is mediated *via* both aquaporins and K^+ channels. However, exposure to $CoCl_2$ and DIDS exhibited no appreciable effects on Se uptake, which would accordingly tend to indicate that the uptake of SeOMet and SeMet is associated with neither Ca^{2+} nor anion channels.

Depriving plants of S significantly increases the uptake of selenate, whereas P starvation induces significant increases in selenite uptake (Li et al., 2008). And there is a competition between selenate and sulfate for uptake by roots (de Souza Cardoso et al., 2022). In the present study, we found that neither S nor P deficiency had the effect of promoting the uptake of SeMet or SeOMet by rice roots (**Table 2**), which might indicate that the uptake of organic Se is independent of sulfate or phosphate transporters. Conversely, under P-deficient conditions, a slight reduction was observed in the root uptake of Se as SeOMet (**Table 2**). In this regard, phosphorylation is one of the factors associated with the regulation of K^+ channel activity (Yu et al., 2006), and thus, a reduction in SeOMet uptake under P-deficient conditions is attributable to diminished K^+ channels activity, which needs to be confirmed by further in-depth molecular studies.

The translocation of selenomethionine and selenomethionine-oxide from roots to shoots

In rice exposed to different sources of Se for 48 h, we found that the proportions of Se distributed in the shoots of rice supplied with the two assessed organic forms of Se ranged from 25.2 to 31.8% (**Table 2**), which are lower than the values we previously recorded in plants supplied with selenate, although slightly higher than those in plants treated with selenite (Huang et al., 2015). In this regard, the findings of several studies on rice and wheat indicated that most of the selenate taken up by roots is subsequently translocated to the shoots (Wang et al., 2015). Conversely, having been absorbed by roots, selenite is rapidly converted to organic forms, such as SeMet, MeSeCys, and SeOMet, which reduces mobility (da Silva et al., 2020). Sulfate transporters such as Sultr2;1, Sultr3;5, and Sultr1;3, are the main transporters involved in the translocation of selenate from roots to shoots (Maruyama-Nakashita, 2017; Mehdawi et al., 2018), whereas phosphorus transporters such as OsPT8 transport selenite in plants (Song et al., 2017). Furthermore, Zhang et al. (2019) found that NRT1.1B, a member of the peptide transporter family, mediates the transport activity of SeMet, whereas in maize, Wang K. et al. (2020) recorded that the values of Se distribution in shoot (%) decreased in the order of selenate treatment > selenite treatment > SeMet treatment when supplied as 0.01 mg L^{-1} Se and in the order of selenate treatment > SeMet treatment > selenite treatment when plants were supplied with 0.1 mg L^{-1} Se. Moreover, Kowalska et al. (2020) found that the translocation of Se from the roots to leaves of lettuce supplied with SeMet was 3.65 times higher than that in lettuce supplied with selenite. These phenomena can presumably be attributed to the differing capacities of the transporters of different Se chemical forms. In the present study, no significant difference was found in the transfer factor

of Se from rice roots to shoots between SeMet and SeOMet (**Figure 2D** and **Table 2**), indicating the similar transportation ability of SeMet and SeOMet in rice plants; while the lowest transfer factor at 18 h might be due to the lowest transpiration rate when treated for 18 h (5:00 am), since the transportation of micro-element from roots to shoots is mainly driven by transpiration (Van der Vliet et al., 2007). Furthermore, we also found that the contents of Se were higher in the shoots of plants supplied with SeMet than in those of plants receiving SeOMet treatment when exposed for up to 26 h (**Figure 2C** and **Table 3**), although the differences were found to gradually diminish with a prolongation of exposure (**Figure 2C** and **Table 2**). This effect of exposure time on organic Se accumulation in shoots is conceivably associated with the transformation of Se in rice plants.

In addition, we established that the uptake and translocation of Se by rice plants maintained in the growth medium treated with both SeMet + SeOMet were considerably lower than the theoretical quantities (**Table 3**), indicating an interaction between SeMet and SeOMet when both Se forms are supplied simultaneously. In this regard, previous studies have reported non-additive effects in the uptake and translocation of different Se chemical forms, with the coexisting selenite being found to inhibit selenate uptake and translocation (Li et al., 2008; Wang et al., 2019). Similarly, in the present study, we found that the presence of SeOMet appeared to suppress the uptake and translocation of SeMet in rice. We speculate that these observations could be explained in terms of a preferential absorption by plants, whereby an optimal absorption strategy is adopted based on intrinsic synergistic activities in response to mixed supplies of different Se chemical forms, thus conserving energy required for subsequent Se assimilation (Versini et al., 2016). However, this needs to be verified in further studies.

Conclusion

In this study, we demonstrated that the uptake and translocation of Se by rice were significantly influenced by both Se chemical forms and treatment time. Compared with inorganic forms, organic Se exhibited a higher uptake potential over the course of a 1 h exposure, with recorded uptake capacity (V_{max}) values declining in the order of SeMet > SeOMet > selenite > selenate. Furthermore, analysis of the time-dependent kinetics of organic Se uptake by roots revealed that, regardless of the duration of exposure in Se-treated growth media, the uptake of SeMet was invariably higher than that of SeOMet, whereas difference between the uptake of these two forms narrowed with time. A similar tendency was detected with respect to the Se in rice shoots. In addition, examination of the effects of selected inhibitors on Se uptake indicated that SeOMet uptake is an energy-dependent symport process and that SeOMet could be imported by rice roots *via* aquaporins and K^+ channels. In contrast, the uptake of SeMet by roots appears to be mediated primarily *via* aquaporins. We also found that when simultaneously supplied with both SeOMet and SeMet, SeOMet appeared to inhibit the uptake and translocation of SeMet.

Data availability statement

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

Author contributions

QW, YW, and HL designed the research and wrote the manuscript. QW performed the experiments. LK helped in data analysis. QH helped in sample determination. All authors read, reviewed, and approved the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.970480/full#supplementary-material

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Foliar selenium fertilization alters the content of dietary phytochemicals in two rocket species

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Biofortification is the process that aims to enrich crops in micronutrients and valuable compounds. Selenium (Se) biofortification has particularly attracted increasing interest in recent times due to the growing number of individuals suffering from Se deficiency. Selenate and selenite are the Se forms most frequently administered to crops. In this study, Se was applied foliarly as selenate at 2.5, 5, or 10 mg per plant to two rocket species, *Diplotaxis tenuifolia* and *Eruca sativa*, grown in soil and the effects in terms of Se enrichment and content of primary and secondary metabolites were comparatively analyzed. We also compared our results with those obtained previously when selenate was supplied to the same species in hydroponics by addition to the nutrient solution. In most cases, the results were the opposite. In *E. sativa*, foliar Se treatment was more effective in promoting Se accumulation, sulfur (S), cysteine, and glucosinolates. No significant effect of Se was evident on total phenolic content, but there were individual phenols. Among amino acids, the content of proline was increased by Se, perhaps to counteract osmotic stress due to high Se accumulation. In *D. tenuifolia*, the content of S and cysteine decreased under Se treatment, but the amount of glutathione was steady, suggesting a preferred assimilation of cysteine toward the synthesis of this antioxidant. Consistent, the content of methionine and glucosinolates was reduced. The content of total phenolics was enhanced only by the low Se dosage. In both species, selenocysteine (SeCys) was identified, the content of which was higher compared to plants grown hydroponically. Concluding, most metabolic differences between rocket species were observed at high Se supplementation. Low Se foliar fertilization was effective in an enriching rocket in Se without affecting other phytochemicals. However, the Se dosages sufficient for biofortification could be even lower, as the Se

concentration in rocket treated with 2.5 mg Se per plant was still very high and the edible part should not be eaten undiluted. Also, a single method of Se supplementation does not appear to be optimal for all plant species or the same species, as the metabolic responses could be very different.

KEYWORDS

rocket, biofortification, selenate, sulfur, glutathione, glucosinolates, amino acids, phenolics

Introduction

Biofortification is the process of adding vital nutrients and health-promoting compounds to crops to improve their nutritional value and enrich the diet of vulnerable populations who frequently have a plant-based diet (White and Broadley, 2009; Zhao and McGrath, 2009; Wu et al., 2015). An emerging area of research focuses on strategies that aim to increase the content of selenium (Se) in staple crops and other vegetables containing low amounts of this element in their edible parts (Schiavon et al., 2020). An estimated 1 billion people have a sub-optimal Se intake in the diet (Combs, 2001), and this number is expected to increase in the future due to the impact of climate change on agriculture (Jones et al., 2017). The resulting Se-biofortified crops can additionally be enriched in other phytochemicals, such as minerals and antioxidant constituents, creating high value vegetables that offer a variety of benefits to consumers (Newman et al., 2019; D'Amato et al., 2020; Schiavon et al., 2020).

Selenium is an essential micronutrient for humans, and the recommended intake is of 55–70 μg per day (World Health Organization, 2009; USDA–ARS, 2012); it is also essential for several animals and microorganisms (Kieliszek, 2019), while its role is different for plants being non-essential (Schiavon and Pilon-Smits, 2017). Plants uptake Se from soil and can transform the inorganic Se into the organic forms, namely the amino acids selenocysteine (SeCys) and selenomethionine (SeMet), but do not possess specific mechanisms for their further insertion in selenoproteins with critical roles in metabolism (White, 2016, 2018). Rather, Se-amino acids are misincorporated in proteins in place of their sulfur (S) analogs cysteine (Cys) and methionine (Met), thus causing protein misfolding and loss of function (Sabbagh and Van Hoewyk, 2012; Van Hoewyk, 2013). In addition, Se compounds at high concentration prompt oxidative stress in cells due to reactive oxygen species (ROS) overgeneration and disruption of reactive nitrogen species (RNS) that leads to protein tyrosine nitration (Kolbert et al., 2016; Gupta and Gupta, 2017). On the other hand, Se at low concentration is recognized as beneficial for many plants by stimulating their growth and antioxidant systems (Chauhan et al., 2019).

Different agronomic and genetic biofortification approaches can be used to increase Se concentration in crops and their success relies on multiple factors, such as the form and dosage of Se applied, the mode of Se administration, the crop species and variety, and the plant growth system (soil or hydroponics) (Ros et al., 2016; Bañuelos et al., 2017). Plants can absorb different forms of Se, either inorganic (e.g., selenate, selenite) or organic (e.g., Se-amino acids). However, inorganic Se salts are more commonly employed in biofortification programs (Schiavon et al., 2020), but they must be applied in small quantities, and for this reason they are often added to fertilizers that act as carriers of Se (Ramkissoon et al., 2019). Selenium fertilizers can be applied to soil to increase the amount of Se available to plants; this method is relatively inefficient: only 12% of soil-applied Se fertilizers were absorbed via root uptake in a study by Broadley et al. (2010). Alternatively, foliar Se administration to plants grown in soil or the addition of Se to the nutrient solution within hydroponic systems could be exploited for biofortification; both methods offer the advantages of fast Se uptake and assimilation by plants, and avoid immobilization processes of Se compounds that may happen in soil. The application of Se via foliar spray also prevents the need of Se root-to-shoot translocation to the edible aboveground organs (Ros et al., 2016; Ramkissoon et al., 2019). The use of Se-laden material derived from Se hyperaccumulator plants as a green manure or growing crops in Se-rich soils are other, but still very limited, options (Bañuelos et al., 2015, 2017; Schiavon et al., 2020).

Plants with the potential to accumulate appreciable amounts of Se in their edible parts are regarded as potential candidates for successful biofortification. Crops belonging to the Brassicaceae are interesting in this respect: they are defined as secondary Se accumulators, based on their capacity to accumulate and tolerate up to 1,000 μg Se g^{-1} d.wt. (White, 2018). These plants have high S content in their tissues, which besides essential S compounds included secondary S compounds, such as glucosinolates (GLS), which play defensive roles against herbivores (Agerbirk and Olsen, 2012; Jørgensen et al., 2015). These compounds also exert protective roles in humans (Melrose, 2019). Owing to the chemical similarity of S to Se, the administration of Se to plants could result in a decrease in S content and, in turn, depletion of primary

and secondary S compounds (Robbins et al., 2005; Schiavon et al., 2013; Bachiega et al., 2016). However, sometimes Se may also stimulate the S uptake and assimilation pathway, leading to higher S levels. In this respect, contrasting results have been reported, even within the same plant species, when using different experimental setups for biofortification (Schiavon et al., 2016).

Previously, we evaluated the effect of Se on the capacity of two rocket species (perennial wall rocket, *Diplotaxis tenuifolia* (L.) DC., and annual garden rocket, *Eruca sativa* Mill.) Grown in hydroponics to accumulate Se depending on interactions with S uptake and assimilation (Dall'Acqua et al., 2019). We also assayed the effect of Se addition to the nutrient solution on the synthesis and accumulation of GLS, phenolic compounds and amino acids. In this study, we aimed to evaluate the effect of different selenate dosages applied via foliar spray on the same rocket species, but cultivated in soil pots, to investigate potential differences in Se accumulation and metabolic-related outcomes depending on the type of Se administration. Indeed, despite the cultivation of plants in hydroponics offers several advantages (e.g., water saved, growth controlled over climate changes, optimal use of nutrients, reduced pests and diseases, and absence of competition with weeds), it is very expensive to manage and requires investment, thus it is not affordable in poor countries where biofortification programs should be more extensively conducted.

We again used *E. sativa* and *D. tenuifolia* for consistency, and because these species differ in the content of health-promoting phytochemicals, have a wide distribution, and are of increasing importance after the circulation of the ready-to-use salads in the vegetable retail markets (Heimler et al., 2007; Caruso et al., 2020). The species *E. sativa*, in particular, has been artificially selected and this may have led to some biochemical and physiological differences with *D. tenuifolia*.

Materials and methods

Experimental setup

Seeds of *E. sativa* and *D. tenuifolia* (Corona sementi, Mortegliano, UD, Italy) were sown in 1 L-pots placed inside a greenhouse under natural light conditions (April to May, average day/night temperature 18/15°C and photoperiod 14/10 h). The pots were filled with peat, soil, and perlite in the ratio 60:30:10, watered twice a day and each contained a germinated plant. Once plants were 6 weeks old, they were divided in four groups (10 pots per group) containing 10 plants each. Three of these groups received a unique foliar application of selenium in the form of sodium selenate (Na_2SeO_4) at dosages of 2.5, 5, or 10 mg per plant. Se-containing solutions differing in selenate concentration (250, 500, and 1,000 mg/L) were prepared and sprayed on the leaves in order to apply them at the same volume (10 ml) to each plant. Plants were quite similar in leaf size, but to avoid dripping from the leaves we applied the selenate solution in two times (5 ml each time) at 1 h interval. One group of plants was sprayed with an equal volume of water and used as a control. During foliar Se treatment, the soil surrounding the plants was covered to avoid any contamination with Se. Plants were harvested 10 days after the Se treatment was applied, washed with distilled water and dried with blotting paper. Specifically, leaves from each plant were immersed in water for about 5 min and then rinsed two times under running distilled water. For roots, at least 5 min were first required to gently clean them from the soil particles. Then, they were subjected to the same procedure as leaves. Six plants per treatment were divided into roots and shoots and their fresh weight was measured individually. The plant material was then placed inside a drying oven for 2 days at 70°C to measure the dry weight. The leaves and roots of the remaining plants were frozen in liquid nitrogen and kept at −80°C for further analyses. The experimental design for plant growth was randomized (the pots were re-arranged three times a week) and the entire experiment was replicated two times. Data were not pooled together from the two experiments to get means, but the trend of Se, S, and metabolic compounds was confirmed by the second experiment.

Determination of total selenium, carbon, nitrogen, and sulfur in soil

Samples of soil dried at room temperature were analyzed for carbon, nitrogen, and sulfur contents using an elemental analyzer (Vario MACRO CNS, Hanau, Germany). For total Se determination, dried soil samples were extracted with HNO_3/HCl (ratio 1:3v/v) and warmed until boiling for 30 min under agitation. Samples were then filtered (0.45 μm, Millipore), and the quantification of Se was performed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Fassel, 1978). Analyses were conducted in triplicates.

Determination of total selenium and sulfur in plants

Leaf and root tissues of rocket plants were dried for 48 h at 80°C and further digested using nitric acid according to the method reported by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectroscopy was used according to the protocol by Fassel (1978) to determine each digest's Se and S elemental concentrations, using appropriate standards and quality controls. Analyses were conducted in triplicates (1 replicate = 1 plant).

Identification and quantification of glucosinolates

Glucosinolates were extracted from rocket leaves according to the protocol reported by Schiavon et al. (2016). To prevent myrosinase activity in the samples, glucosinolates were extracted from 6 g of leaves boiled for 4 min in 18 ml of a methanol/water solution (ratio 70:30, v/v). Sinigrin (1.26 mg/ml) was added as internal standard to this solution. To achieve the complete glucosinolates extraction, leaf material residual after sample filtration was re-extracted using 70% (v/v) methanol for 4 min. The two extracts from each sample were further combined and purified through a Solid-Phase Extraction (SPE) column (0.8 cm × 4 cm, Agilent Technologies) equipped with 0.256 g of an ion-exchange resin (DEAE-SEPHADEX-A25) imbibed in 4 ml of a 0.5 M Na-acetate buffer solution (pH = 5). The column was washed with 1 ml deionized H_2O and then loaded with 2.5 ml extract containing the internal standard (Sinigrin). The further purification steps were performed according to the protocol reported by Schiavon et al. (2016).

The analysis of glucosinolates was performed in High Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS) on a Varian LCMS 500 Ion Trap equipped with Electrospray Ionization (ESI) as a source operating in positive ion-mode. The analysis of the fragmentation patterns of spectra shown in **Supplementary Table 1** was realized through the Turbo Detection Data Scanning (TDDS) function. The chromatographic separation was performed in an Agilent 1,260 Liquid Chromatography (LC) system using a column Eclipse XDB C-8 5 μm 2.1 mm × 150 mm as described by Schiavon et al. (2016). For the quantification of glucosinolates, glucoerucin was used as a reference standard at different concentration levels. Analyses were performed on three biological replicates (1 replicate = 1 plant).

Determination of low molecular weight thiol compounds

Frozen leaf material (250 mg) was ground in liquid nitrogen with 0.1 N HCl and 1 mM Ethylenediaminetetraacetic acid (EDTA). Extracts were centrifuged at 10,000 g for 10 min and then analyzed for low-molecular-weight (LMW) thiol contents. Extracts (50 μL) were further derivatized using 7-Fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F) fluorophore (Sigma-Aldrich, St. Louis, MO, United States). Low-molecular-weight thiols (cysteine and total glutathione) were separated by isocratic HPLC according to Masi et al. (2002). The mobile phase was 3% methanol in 75 mM NH_4^+ formiate, pH 2.9. Analyses were performed on three biological replicates (1 replicate = 1 plant).

Quantification and identification of free amino acids

Free amino acids were determined in rocket leaves instead of total amino acids to get preferential information about metabolism rather than the function of gene expression. Extraction of free amino acids, including Se-amino acids, was obtained from three replicates of frozen rocket leaves (500 mg) using 0.1 M HCl (1:4, w/v). The extracts underwent centrifugation at 4°C for 10 min at 10,000 g. The supernatants were collected and filtered at 0.45 μm (Millipore). Qualitative and quantitative analyses of amino acids were realized through HPLC-MS using a Varian Liquid Chromatography - Mass Spectrometry (LC-MS) 500 equipped with a ZORBAX Eclipse Plus AAA column (3.5 μm × 3 mm × 150 mm) as described by Schiavon et al. (2016). The identification and quantification of the amino acids in the extracts were attained via Ion Trap Mass Spectrometry (Varian 500 MS) coupled to the HPLC system, by comparison with appropriate standards and analysis of the fragmentation patterns of spectra (data not shown) through the TDDS function. For the identification and quantification of the amino acids, the reference standards consisted of these amino acids: Alanine, Arginine, Asparagine, Glutamine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, Valine, Selenomethionine, Selenocysteine, and Se-Methyl-Selenocysteine. The free amino acids whose content was below the detection limit are not reported in **Table 1**. Analyses were performed on three biological replicates (1 replicate = 1 plant).

Identification and quantification of polyphenols

Extraction of polyphenols from three replicates of frozen rocket tissues was performed using methanol: water (1:1, v/v) solution in ultrasonic bath for 15 min. The ratio of plant material to the mixture was 1:10 (w/v) and extracts were filtered at 0.45 μm (Millipore). Validation of the extraction procedure was realized by measuring the recovery percentage of chlorogenic acid and rutin in replicates of leaf samples.

Qualitative and quantitative analyses of polyphenols were realized both via HPLC-MS and High Liquid Chromatography with Diode Array detector (HPLC-DAD). For the separation of polyphenols, an Eclipse Plus C-18 column (3.5 μm × 2.1 mm × 150 mm, Agilent) was used in the HPLC system Varian 212 at 35°C as reported in Schiavon et al. (2016). The identification and quantification of the principal polyphenols in the extracts were conducted via Ion Trap Mass Spectrometry (Varian 500 MS) coupled to the HPLC system, by comparison with appropriate standards (chlorogenic acid for phenols, rutin for flavonoids) and analysis of the fragmentation

patterns of spectra (Supplementary Table 2) through the TDDS function. Electrospray Ionization was used as a source in negative ion-mode and the mass range considered was within 50–3,500 uma. Each sample's volume injected was equal to 10 μL. Analyses were performed on three biological replicates (1 replicate = 1 plant).

Statistical analysis

Analysis of variance (ANOVA) was performed using the SPSS software, and was followed by pair-wise *post-hoc* analyses (Student–Newman–Keuls test) to determine which means differed significantly at $p < 0.05$ (+STD).

Results

Plant growth in response to selenium application

Foliar application of Se at the minimum dosage (2.5 mg Se per plant) increased the fresh leaf and root biomass of both rocket species, while no significant effect on growth was observed when higher Se doses (5 and 10 mg Se per plant) were administered to the plants (Figures 1A,B). The increment in leaf biomass was more pronounced for *D. tenuifolia* (+28.2 vs. 16.6.%), while the root biomass was more enhanced in *E. sativa* (+37.5 vs. 14.9%) (Figures 1C,D). A similar trend was evident for plant dry weight (data not shown). Figure 1E depicts representative rocket plants fertilized with different amounts of Se.

Selenium accumulation and effects on sulfur and thiol compounds

The concentration of Se in leaves and roots of *E. sativa* positively correlated with the amount of applied Se (Figures 2A,B), while in *D. tenuifolia* this type of correlation was determined only when plants received up to 5 mg Se per plant (Figures 2C,D). The application of a higher Se dosage (10 mg per plant) did not further increase the Se content in *D. tenuifolia*, as a plateau was distinctly achieved. The two species contained similar Se concentrations when supplied with 2.5 or 5 mg Se per plant. *E. sativa* accumulated about 2-fold more Se at a higher Se dosage compared to *D. tenuifolia*. In general, plants accumulated approximately 7.5 times more Se in leaves than roots.

TABLE 1 Effects of selenate treatment on the content of selected amino acids in leaves of rocket species (*Eruca sativa* and *Diplotaxis tenuifolia*) grown in soil and treated foliarly with selenate dosages ranging from 0 to 10 mg per plant.

Amino acid (mg/100 g FW)	0	2.5	5	10
***Eruca sativa* Se treatment (mg per plant)**				
Phenylalanine	1.50 ± 0.10b	1.36 ± 0.19ab	1.64 ± 0.14ab	1.84 ± 0.16a
Isoleucine	0.45 ± 0.02b	0.82 ± 0.14a	0.18 ± 0.05c	0.13 ± 0.02c
Leucine	0.42 ± 0.03b	0.65 ± 0.09a	0.20 ± 0.12c	0.08 ± 0.02c
Histidine	2.30 ± 0.39b	3.83 ± 0.50a	4.00 ± 0.37a	4.40 ± 0.28a
Tyrosine	0.14 ± 0.01a	0.18 ± 0.04a	0.15 ± 0.05a	0.18 ± 0.05a
Tryptophan	0.35 ± 0.10a	0.40 ± 0.13a	0.30 ± 0.15a	0.32 ± 0.10a
Arginine	1.53 ± 0.22d	0.85 ± 0.06c	0.45 ± 0.02b	0.27 ± 0.07a
Alanine	5.83 ± 1.84bc	3.77 ± 0.77c	5.64 ± 0.66b	11.46 ± 3.36a
Valine	2.32 ± 0.68b	3.74 ± 0.52a	3.96 ± 0.31a	3.08 ± 0.15ab
Lisine	1.18 ± 0.05a	1.11 ± 0.08a	1.06 ± 0.07a	1.20 ± 0.07a
Proline	18.07 ± 3.44b	34.06 ± 8.61a	21.94 ± 7.17ab	20.55 ± 5.80ab
Methionine	0.15 ± 0.03a	0.11 ± 0.05a	0.14 ± 0.04a	0.17 ± 0.04a
Se-cysteine	0.00 ± 0.00c	5.86 ± 0.64a	5.58 ± 0.13a	4.23 ± 0.51b
***Diplotaxis tenuifolia* Se treatment (mg per plant)**				
Phenylalanine	1.19 ± 0.05b	1.19 ± 0.05b	1.19 ± 0.05b	1.19 ± 0.05b
Isoleucine	0.09 ± 0.02c	0.18 ± 0.01b	0.13 ± 0.02c	0.36 ± 0.08a
Leucine	0.09 ± 0.01b	0.13 ± 0.03 ab	0.11 ± 0.01b	0.20 ± 0.03a
Histidine	3.70 ± 0.26a	4.00 ± 0.39a	3.73 ± 0.13a	4.58 ± 0.19a
Tyrosine	0.17 ± 0.01a	0.17 ± 0.01a	0.17 ± 0.01a	0.17 ± 0.01a
Tryptophan	0.31 ± 0.12a	0.31 ± 0.12a	0.31 ± 0.12a	0.31 ± 0.12a
Arginine	1.20 ± 0.14a	1.33 ± 0.16a	0.95 ± 0.12a	1.17 ± 0.27a
Alanine	6.31 ± 0.67a	6.62 ± 1.14ab	3.66 ± 0.10c	5.03 ± 0.81b
Valine	2.75 ± 0.91a	2.46 ± 0.50a	2.40 ± 0.32a	3.15 ± 0.96a
Lisine	1.20 ± 0.06a	1.17 ± 0.06a	1.08 ± 0.02a	1.10 ± 0.05a
Proline	20.76 ± 6.05a	21.30 ± 1.80a	14.25 ± 3.08ab	13.81 ± 1.71b
Methionine	0.24 ± 0.04a	0.21 ± 0.04a	0.25 ± 0.03a	0.06 ± 0.02b
Se-cysteine	–	4.89 ± 0.74a	4.89 ± 0.74a	4.89 ± 0.74a

Different letters along rows indicate significant differences ($p < 0.05$, ±STD) among treatments.

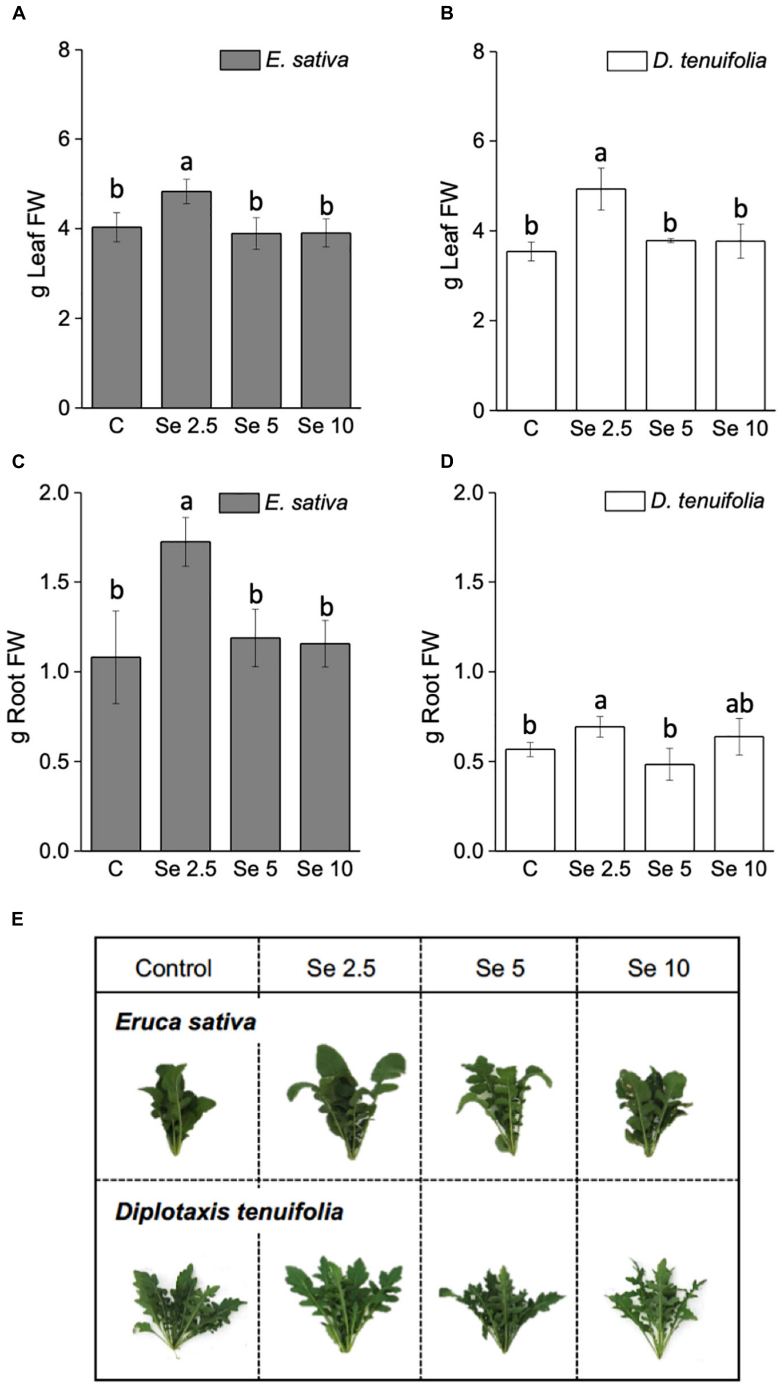

FIGURE 1
Fresh weight (FW) of leaves **(A,B)** and roots **(C,D)** of *E. sativa* and *D. tenuifolia* plants grown in soil and subjected to foliar fertilization with selenate dosages ranging from 0 to 10 mg per plant. The FW reported is the average FW of each leaf (±SD, $n = 6$). Different letters above bars indicate significant differences between the means ($p < 0.05$). **(C,E)** Images of representative rocket species fertilized with selenate.

The trend of S content in response to Se application was contrasting between the two species (Figures 3A,B). With respect to *E. sativa*, the S leaf content increased with the increase of applied Se, while a decline was evident in the roots. In contrast, the Se administration to *D. tenuifolia* caused a significant depletion of S. This effect was determined in leaves fertilized with 10 mg Se per plant, and in roots at any Se dosage applied (Figures 3C,D).

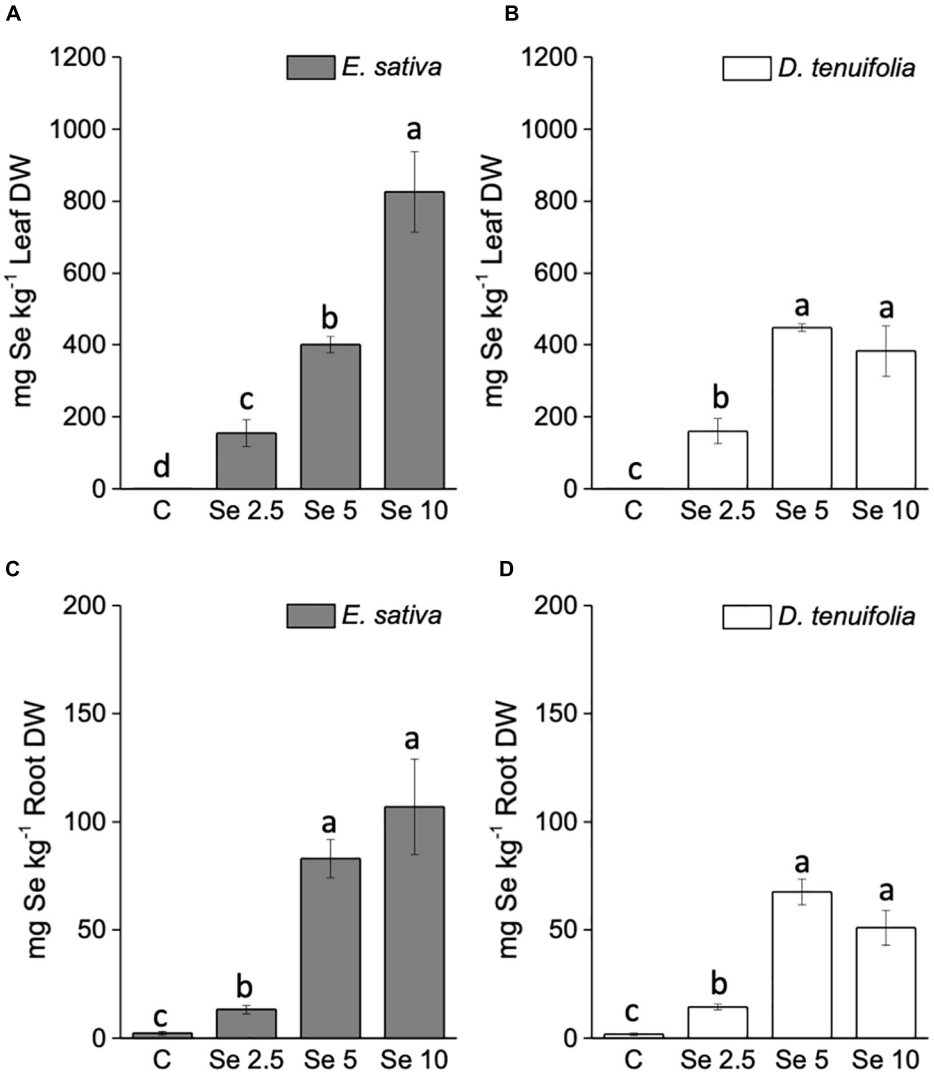

FIGURE 2
Selenium concentration in leaves **(A,B)** and roots **(C,D)** of *E. sativa* and *D. tenuifolia* plants grown in soil and subjected to foliar fertilization with selenate dosages ranging from 0 to 10 mg per plant. Data shown are the mean ± SD of three replicates. Different letters above bars indicate significant differences between the means ($p < 0.05$).

The Se:S ratio was overall higher in leaves than in roots (Figures 3E,F). The leaf Se:S ratio was comparable between *E. sativa* and *D. tenuifolia* at any Se dosage applied, but the values in roots were greater for *E. sativa* plants supplemented with 10 mg Se per plant. This is because, although S decreased in both species, *E. sativa* contained more Se in roots than *D. tenuifolia*.

In line with the trend of S accumulation, the leaf content of the amino acid cysteine (Cys) increased in *E. sativa* plants sprayed with Se (Figure 4A), whereas it was depleted in D. *tenuifolia* (Figure 4B). The leaf content of glutathione (GSH) was almost unchanged by Se fertilization in both rocket species (Figures 4C,D).

Effects of selenium application on the amount of total and individual glucosinolates

The total glucosinolate (GLS) content did not vary in response to low (2.5 mg Se per plant) Se application in either of the rocket species (Figures 4E,F). However, the supplementation of higher Se dosages led to an increase of GLS in *E. sativa*, and a decrease in *D. tenuifolia*.

The GLS identified in both species are listed in Supplementary Table 1 and mainly consisted of aliphatic GLS derived from methionine. We did not detect any Se-GLS, though they have been identified in other species

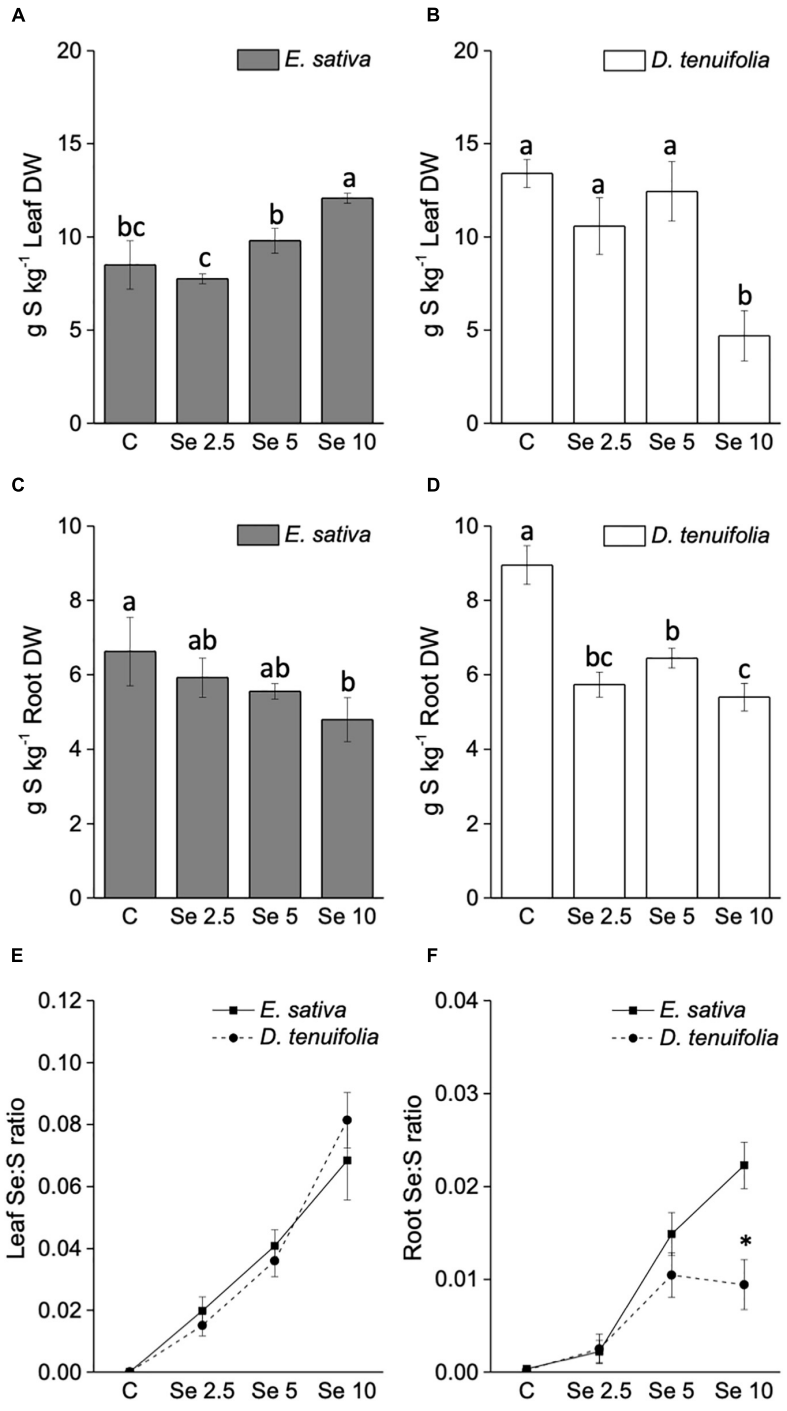

FIGURE 3
Sulfur concentration in leaves **(A,B)** and roots **(C,D)** of *E. sativa* and *D. tenuifolia* plants grown in soil and subjected to foliar fertilization with selenate dosages ranging from 0 to 10 mg per plant. Se:S ratio of leaves **(E)** and roots **(F)** of the two rocket species. Data shown are the mean ± SD of three replicates. Different letters above bars indicate significant differences between the means ($p < 0.05$). In the line graph, the asterisks indicate significant differences in the root Se:S ratio between the two rocket species.

(McKenzie et al., 2019). The most abundant was DMD (Dimeric-4-mercaptobutyl)-GLS, followed by glucoraphanin, glucosativin and glucoerucin (**Figures 5A–D**). In *E. sativa*, the content of most GLS (glucoerucin, glucoraphanin, DMD-GLS, glucoalissin, methoxy glucobrassicin, glucosativin, and neoglucobrassicin) was enhanced by Se fertilization,

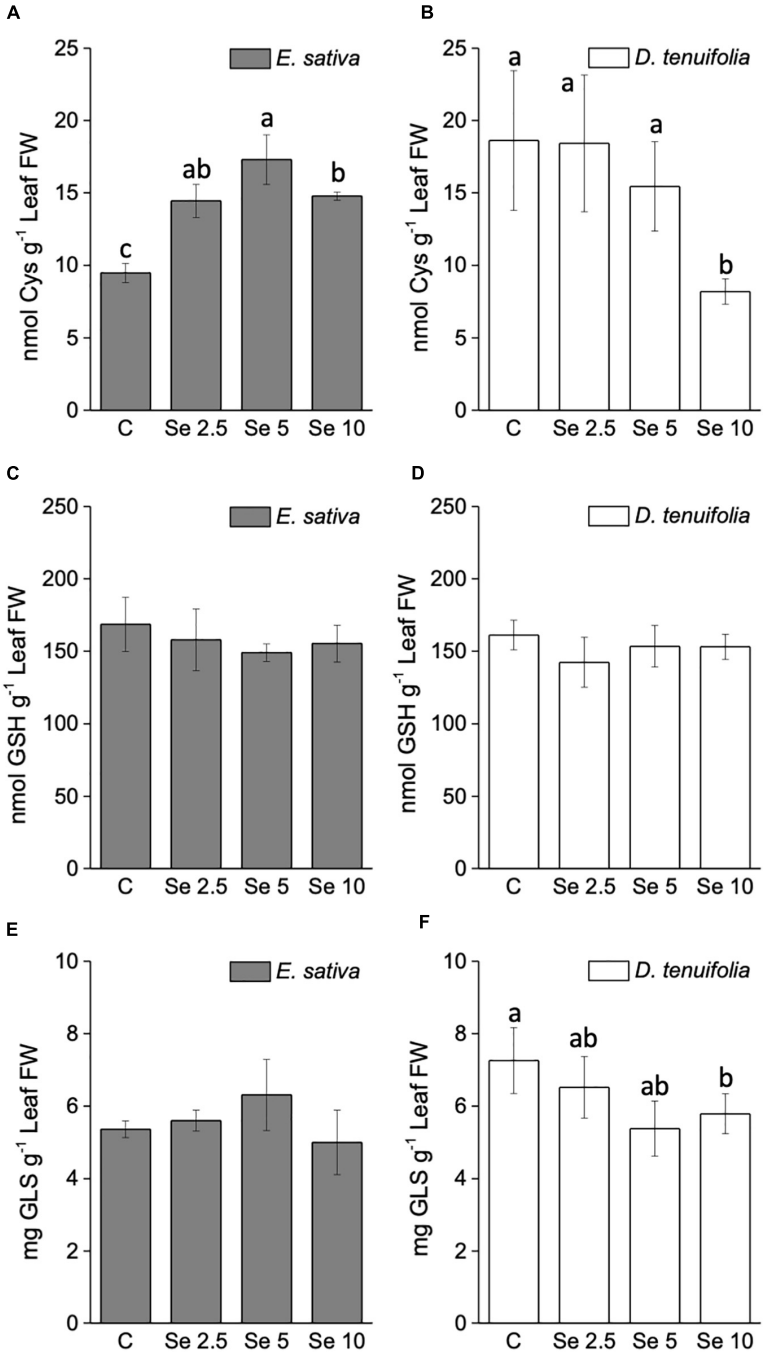

FIGURE 4
Content of cysteine **(A,B)**, total glutathione **(C,D)** and total glucosinolates **(E,F)** in leaves of *E. sativa* and *D. tenuifolia* plants grown in soil and subjected to foliar fertilization with selenate dosages ranging from 0 to 10 mg per plant. Data shown are the mean ± SD of three replicates. Different letters above bars indicate significant differences between the means ($p < 0.05$).

especially when Se was supplemented to plants at 5 or 10 mg per plant (Figures 5A–F). Neoglucobrassicin was more abundant in *E. sativa* than *D. tenuifolia* (Figure 5G), and the content of hydroxyglucobrassicin did not vary in response to Se administration (Figure 5H). In *D. tenuifolia*, however, the glucoerucin content was higher than in *E. sativa*, but the application of 5 or 10 mg Se per plant substantially decreased it (Figure 5D). A similar decreasing trend was observed in *D. tenuifolia* for DMD-GLS, glucosativin, glucoalissin,

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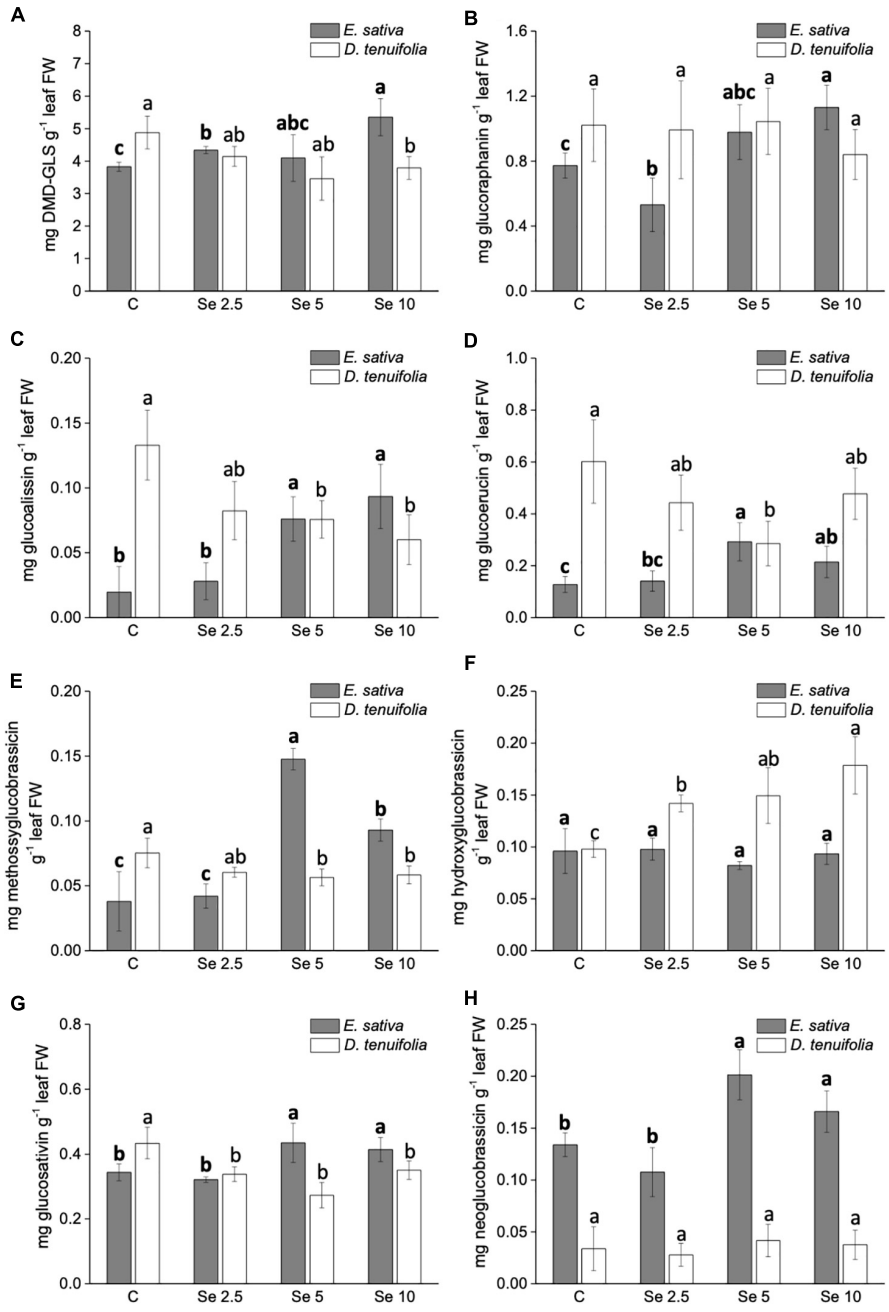

FIGURE 5
Effects of selenate treatment on the content of individual glucosinolates, i.e., DMD-GLS **(A)**, glucoraphanin **(B)**, glucoalissin **(C)**, glucoerucin **(D)**, methossiglucobrassicin **(E)**, hydroxyglucobrassicin **(F)**, glucosativin **(G)**, and neoglucobrassicin **(H)**, identified in *Eruca sativa* and *Diplotaxis tenuifolia* plants grown in soil and subjected to foliar fertilization with selenate dosages ranging from 0 to 10 mg per plant. Data shown are the mean ± SD of three replicates. Different letters in bold above bars indicate significant differences between the means ($p < 0.05$) of values referred to *E. sativa*, while different letters not bolded indicate significant differences between the means ($p < 0.05$) of values referred to *D. tenuifolia*. DBM-GLS = Dimeric-4-mercaptobutyl glucosinolate.

and methoxy glucobrassicin (Figures 5A,C,E,F), while the glucoraphanin and neoglucobrassicin contents were almost unchanged (Figures 5B,G). The only GLS of *D. tenuifolia* whose content increased after Se supplementation was hydroxyglucobrassicin (Figure 5H).

Effects of selenium application on the content of free amino acids

The foliar fertilization with Se determined different effects on the abundance of single amino acids in both species (Table 1).

In *E. sativa*, the content of leucine, isoleucine and proline was increased by the low Se dosage (2.5 mg Se per plant). Leucine and isoleucine were then reduced by higher Se dosages compared to the Se untreated plants. Histidine and valine were more accumulated with any dosage of Se applied, while phenylalanine and alanine were only by 10 mg Se per plant. In contrast, the amount of arginine was depleted by Se fertilization, while no effect was evident on tyrosine, tryptophan and lysine. In *D. tenuifolia*, the content of leucine, isoleucine and histidine increased with increasing Se dosages. Proline, alanine and lysine were decreased in plants sprayed with 5 or 10 mg Se per plant. The amount of valine and arginine and of the aromatic amino acids tyrosine and tryptophan was not affected by Se, but phenylalanine was more abundant in plants fertilized with 10 mg Se per plant.

Among Se amino acids, only SeCys was detected and its content was comparable in *E. sativa* and *D. tenuifolia*. Notably, the SeCys content declined in both species with high Se supplementation.

Effects of selenium application on the amount of single and total polyphenolic compounds

Variation in the leaf content of single polyphenols identified in *E. sativa* and *D. tenuifolia* is reported in **Table 2**. These compounds consisted mainly of flavonoid derivatives. Specifically, glycosylated derivatives of kaempferol and isorhamnetin, often esterified with phenylpropanoid acids, were dominant in *E. sativa*, especially kaempferol-3,4′-diglucoside and kaempferol-3-sin a poil triglucoside-7′-glucoside, while derivatives of quercetin and kaempferol abounded in *D. tenuifolia*. Only two compounds were shared by both species, i.e., isorhamnetin-3,4-diglucoside and quercetin-3-glucoside.

In the analyzed samples, *E. sativa* contained more phenolic compounds than *D. tenuifolia*. However, while the total content of phenols was not substantially affected by Se fertilization in *E. sativa* plants, a substantial increase over control plants was evident in *D. tenuifolia* administered with Se, especially when supplied at the low dosage (2.5 mg per plant, +46%). These results mainly derive from the different behavior of individual phenol compounds in the two rocket species. In *E. sativa*, the content of kaempferol-3-sinapoyl diglucoside-7′-glucoside, quercetin-3-glucoside, kaempferol-3-snap sophoroside-7′-glucoside and quercetin-3-glucoside-3′-(6-sinapoyl-glucoside) slightly increased, while isorhamnetin-3-glucoside and kaempferol-3-(2-sinapoyl-glucoside)-4′-glucoside decreased. In *D. tenuifolia*, phenol compounds increased with Se application except for quercetin-3,3′,4′-triglucoside, which was almost unchanged, and quercetin-3-(2-feruloilglucoside)-3′-(6-sinapoil-glucoside)-4′-glucoside whose content conversely decreased.

Content of selenium, nitrogen, carbon, and sulfur in soil after foliar selenium fertilization

The soil used to fill the pots initially contained a very low Se concentration (<0.5 mg kg^{-1}) (**Table 3**). After the plant treatment with Se, we observed a weak increase in soil Se concentration, though it generally remained below 2.5 mg kg^{-1}. The content of S in soil pots where *E. sativa* was cultivated decreased with increasing dosage of Se applied, and conversely increased in the soil where *D. tenuifolia* was grown. With respect to the content of N, inorganic C, and organic C, no significant differences were evident between pots containing plants fertilized with Se and pots with unfertilized plants.

Discussion

In this study, we evaluated the effect of foliar application of selenate at different dosages on the accumulation of Se and on the content and profile of beneficial phytochemicals in two species of rocket. The need to study the content of different plant constituents after Se biofortification is to establish if the potential changes induced by the increased amount of Se in plants depending on the method of Se application could significantly impact other health-promoting nutritional components.

Using different methods of Se supplementation to the same plant species can result in distinct outcomes (Schiavon et al., 2016). In previous work, Se was applied to *E. sativa* and *D. tenuifolia* plants grown in hydroponics by adding selenate to the nutrient solution (Dall'Acqua et al., 2019). In that case, we found that such a method of Se administration was effective in enriching both species in Se, but elevated Se dosages (≥10 μM) determined a too high Se accumulation in leaf tissues resulting in plant material that cannot be considered completely safe. Furthermore, *D. tenuifolia* accumulated more Se than *E. sativa* likely because of greater S uptake that warranted a high abundance of S and S-containing compounds (Cys, GSH, and GLS) in plant tissues. In the present study, we did not observe differences in Se accumulation between *E. sativa* and *D. tenuifolia* when plants were foliarly supplied with low Se dosages (2.5 and 5 mg per plant), but at high Se supply (10 mg per plant) *E. sativa* revealed to accumulate significantly more Se. A possible explanation could lie in the fact that the leaf area of *E. sativa* plants is larger compared to *D. tenuifolia*, and thus would result in a greater number of transcuticular pores and stomata on the leaf surface that mediate the entry of Se into the mesophyll tissue (Reynoud et al., 2021).

Selenium was mainly determined in the leaf organs of both species, consistent with the method of Se supplementation, but was also partly accumulated in the root apparatus, indicating

the capacity of rocket to efficiently translocate Se across the phloem. So far, little is known about the redistribution of selenate through the phloem (Trippe and Pilon-Smits, 2021), but the high-affinity sulfate transporter SULTR1;3 that localizes to phloem companion cells in both roots and shoot and is involved in the delivery of sulfate from source to sink organs (Yoshimoto et al., 2002) might have a role in Se mobilization, as its expression was recently reported to be upregulated in response to selenate treatment in wheat (Boldrin et al., 2016). Se transportation from leaves to roots was previously reported in other plant species like radish (Schiavon et al., 2016) and carrot (Kápolna et al., 2009). We exclude that Se in roots derived from Se deposition in soil after selenate treatment, as no significant variation in the natural content of Se in soil was determined. It is likely that conveying Se to the roots could be a strategy of rocket plants to limit Se accumulation in the leaves, thus reducing the toxicity of excess Se in photosynthetic tissues. Indeed, many plants tend to accumulate metals and metalloids in the roots to prevent their toxicity and ROS overgeneration in the shoot (Dal Corso et al., 2013).

TABLE 2 Content of phenolic compounds identified in leaves in leaves of rocket species (*Eruca sativa* and *Diplotaxis tenuifolia*) grown in soil and foliarly fertilized with selenate dosages ranging from 0 to 10 mg per plant.

Polyphenol (mg/100 g FW)	0	2.5	5	10
***Eruca sativa* Se treatment (mg per plant)**				
Q-3-glucoside	0.00 ± 0.00a	0.00 ± 0.00a	0.21 ± 0.04a	0.10 ± 0.01a
I-3-glucoside	0.11 ± 0.02b	0.23 ± 0.04a	0.07 ± 0.02b	0.08 ± 0.04b
K-3,4′-diglucoside	6.09 ± 0.98b	5.26 ± 0.91b	6.18 ± 0.44b	4.96 ± 0.25a
I-3,4′-diglucoside	0.49 ± 0.17b	0.53 ± 0.08b	0.41 ± 0.11b	0.44 ± 0.15a
K-3-(2-sinapoil-glucoside)-4′-glucoside	0.46 ± 0.05a	0.41 ± 0.12a	0.30 ± 0.02a	0.14 ± 0.04b
Q-3-glucoside 3′ (6-sinapoilglucoside)	0.00 ± 0.00a	0.09 ± 0.03a	0.24 ± 0.09b	0.09 ± 0.04b
K-3-sinapoyl sophoroside-7′-glucoside	0.15 ± 0.01b	0.38 ± 0.02a	0.38 ± 0.06a	0.21 ± 0.01a
K-3-sinapoil-triglucoside-7-glicoside	2.35 ± 0.17a	2.51 ± 0.19a	3.29 ± 0.27b	2.99 ± 0.16a
Total phenolic compounds	9.65 ± 0.98a	9.41 ± 0.77a	11.08 ± 0.55b	9.12 ± 0.46a
***Diplotaxis tenuifolia* Se treatment (mg per plant)**				
1-Sinapolyglucoside	0.00 ± 0.00a	0.00 ± 0.00a	0.44 ± 0.07a	0.62 ± 0.02a
Q-3-glucoside	0.12 ± 0.01a	0.18 ± 0.06a	0.07 ± 0.03a	0.08 ± 0.04a
Q-3,4′-diglucoside	0.13 ± 0.04b	0.39 ± 0.06a	0.16 ± 0.04b	0.12 ± 0.01b
I-3,4′-diglucoside	0.18 ± 0.02b	0.63 ± 0.09b	1.10 ± 0.20b	0.89 ± 0.18a
Q-3,3′,4′-triglucoside	0.23 ± 0.03b	0.19 ± 0.04b	0.13 ± 0.06b	0.23 ± 0.01a
Q-3,4′-diglucoside 3′ (6-sinapoil-glucoside)	0.00 ± 0.00a	0.00 ± 0.00a	0.19 ± 0.05a	0.11 ± 0.01b
Q-3-(2-ferulok glucoside)-3′-(6-sinapolyglucoside)-4′-glucoside	0.93 ± 0.33a	1.55 ± 0.16a	0.29 ± 0.17b	0.21 ± 0.17b
Total phenolic compounds	1.59 ± 0.36c	2.95 ± 0.23a	0.047 ± 0.22b	0.061 ± 0.22b

Data represent the mean of four biological replicates. Different letters along rows indicate significant differences ($p < 0.05$, ±STD) among treatments.
K, kaempferol; Q, quercetin; I, isorhamnetin.

TABLE 3 Content of total C, N, S and Se in soil where rocket species (*Eruca sativa* and *Diplotaxis tenuifolia*) were grown in soil and foliarly fertilized with selenate dosages ranging from 0 to 10 mg per plant.

Soil element	Time 0	0	2.5	5	10
***Eruca sativa* Se treatment (mg per plant)**					
C (%, w/w)	23.4 ± 0.10	21.88 ± 0.70a	20.85 ± 0.8a	20.76 ± 0.57a	21.51 ± 0.80a
N (%, w/w)	0.60 ± 0.08	0.57 ± 0.05b	0.50 ± 0.04a	0.48 ± 0.07b	0.52 ± 0.06b
S (%, w/w)	0.18 ± 0.01	0.16 ± 0.01b	0.16 ± 0.01b	0.14 ± 0.01ab	0.13 ± 0.00a
Se (mg kg^{-1})	0.02 ± 0.00	0.00 ± 0.00b	1.30 ± 0.36a	1.40 ± 0.20a	1.25 ± 0.13a
***Diplotaxis tenuifolia* Se treatment (mg per plant)**					
C (%, w/w)	23.4 ± 0.10	23.09 ± 0.00a	22.37 ± 0.10a	21.60 ± 0.17a	22.68 ± 0.10a
N (%, w/w)	0.60 ± 0.08	0.53 ± 0.01a	0.52 ± 0.06a	0.51 ± 0.03a	0.57 ± 0.04a
S (%, w/w)	0.18 ± 0.01	0.13 ± 0.01b	0.17 ± 0.01a	0.16 ± 0.01a	0.17 ± 0.02a
Se (mg kg^{-1})	0.02 ± 0.00	0.01 ± 0.00b	1.21 ± 0.30a	1.35 ± 0.27a	1.32 ± 0.35a

The initial concentration of C, N, S and Se is reported in brackets. The soil samples were analyzed in triplicates. Statistical differences are indicated by different letters ($p < 0.05$, ±STD). Comparison are made only between values measured at the end of the experiment.

The low Se dosage (2.5 mg per plant) had positive effects on both the leaf and root biomass of the two rocket species. Such a beneficial effect of Se on plant growth at low concentration is well-known and thought to be associated with cell membrane development, stimulation of photosynthetic efficiency in terms of faster electron transport rate along photosystems and chloroplast development (Schiavon and Pilon-Smits, 2017), and upregulation of antioxidant metabolism (Chauhan et al., 2019). It is noteworthy that the growth of rocket plants was more pronounced when they were cultivated in soil than in the hydroponic setup. Several possible reasons may have caused this difference. First, the plants grown in soil were 1 week older than plants placed in hydroponics; second, the plants raised in hydroponics were transplanted from the agar medium, which may have generated temporary, albeit mild, stress; third, plants within the same pot in hydroponics could have competed for nutrient resources limiting growth; fourth, certain soil rhizosphere microorganisms may have stimulated plant growth and/or contributed to alleviate Se stress (de Souza et al., 1999; White and Broadley, 2009; El Mehdawi and Pilon-Smits, 2012; Winkel et al., 2015; Ye et al., 2020).

The application of the low selenate dosage (2.5 mg Se per plant) through foliar spray also appeared to be a worthy approach to enrich *E. sativa* and *D. tenuifolia* with Se, but still provided a too high amount of this element to consumers. Indeed, the consumption of about only 4–5 g of leaf fresh material derived from *E. sativa* or *D. tenuifolia* plants would meet the daily consumers' requirement for Se, which ranges from 55 to 70 μg. On the other hand, the supply of higher Se dosages (5 or 10 mg Se per plant) to rocket species caused a very high Se accumulation in the shoot, and therefore only little amounts of leaf material from *E. sativa* (<1–1.93 g leaf FW) or *D. tenuifolia* (1.47–2.19 g leaf FW) could be safely consumed. In any case, as the amount of leaf material that is recommended is overall very small, the fresh leaves of either species could be more suitably added to mixed salads for consumption. These results differ compared to those obtained in the hydroponic study, where *E. sativa* accumulated much less Se in leaves, and thus greater consumption of leaf fresh material from this species could be recommended (Dall'Acqua et al., 2019). Perhaps, the accumulation of Se in leaves of *E. sativa* growing in hydroponics was constrained by the Se uptake capacity of the root system and the further Se root to shoot translocation rate. Conversely, in the current study, the direct application of selenate on the leaf surface ensured a faster Se absorption by this species, even though a little part of Se could also have been lost by leaf washing after harvest.

Selenocysteine (SeCys) was the unique Se amino acid determined in both rocket species, as in the hydroponic study. However, the amount of this compound was substantially higher when plants were foliarly fertilized with Se, and values were comparable between *E. sativa* and *D. tenuifolia*. This means that foliar Se fertilization could be more efficient in enriching rocket in this form of organic Se. This is important to note, because organic Se species, such as Se-amino acids, are considered the most efficient form of biofortification (Davis, 2012). The reduction of free SeCys may be due to its increased incorporation into proteins in place of Cys, which apparently was rather used more for the steady synthesis of GSH.

The impact of Se biofortification on the content of S and S-containing compounds (Cys and total GLS) was the opposite in the two rocket species. Similarly to findings obtained in the hydroponic study, Se and Cys were more abundant in *D. tenuifolia* than *E. sativa* under no Se or low Se treatment, and *E. sativa* plants supplied with the high Se dosage exhibited the elevated capacity to re-mobilize S, which was early found to be dependent on the up-regulation of the low affinity sulfate transporter SULTR2;1, involved in Se/S root to shoot translocation (Dall'Acqua et al., 2019). However, here we found the accumulation of Cys increased at any Se dosage applied, thus suggesting that enhanced S assimilation can be induced by foliar selenate treatment. It cannot be ruled out that part of Cys may also derive from the turnover of proteins that removes those abnormal or misfolded (Vierstra, 1996). The enhanced accumulation of Cys in *E. sativa* probably served to prevent a decline in the amount of other essential S-compounds such as Met and GSH, which require Cys as a precursor for their synthesis. Consequently, Met-derived GLS were unaffected by Se treatment. These results are different from those reported in the same species supplemented with Se in hydroponics (Dall'Acqua et al., 2019), where selenate treatment affected the expression of genes involved in the S assimilation pathway, consequently reducing the synthesis of Cys, GSH, Met, and GLS, and indicates that the foliar or soil supplementation of Se can yield to different plant responses.

In *D. tenuifolia*, the Cys content followed an opposite trend compared to *E. sativa* as it was reduced by the high selenate treatment, while S was decreased at any applied selenate dosage. The decrease in S can be due to an effect of foliar Se treatment on S acquisition by roots, as the amount of S in the soil of plants treated with Se was higher than the soil used to grow untreated plants. This result indicates the existence of a long-distance effect of Se applied on leaves on the Se/S root uptake transport systems in this species. Although S decreased in roots, plants treated with 2.5 or 5 mg Se per plant maintained a steady level of S in leaves, ensuring a constant synthesis of Cys. The decrease in Cys content that occurred with high Se treatment was in line with the reduction of S in leaves, but the fact that the GSH content was unchanged suggests that Cys was the preferential precursor for GSH over other compounds. In support of this, the content of Met decreased at the high selenate dosage in *D. tenuifolia*, consistent with the reduction of GLS, most of which were Met-derived.

The reduction of GLS content is a disadvantage of Se fertilization and could have potential ecological implications. GLS are involved in the defense of plants against herbivores

and pathogens and their decrease could limit the plant's capacity to prevent attacks by these organisms, with consequent yield losses. To some extent, the accumulated Se may take over the protective role: even levels as low as 1 mg kg^{-1} dry weight (DW) have already been shown to be protective against generalist herbivores, due to deterrence and toxicity (Hanson et al., 2004). On the other side, GLS are also responsible for the typical bitter taste of rocket. So, Se may reduce the bitterness of wild rocket making it potentially attractive for some consumers.

The Se foliar treatment altered the content and profile of free amino acids and phenolic compounds in the two rockets species, with some differences. Early studies reported changes in the content of such compounds due to Se (Djanaguiraman et al., 2005; Schiavon et al., 2016; Dall'Acqua et al., 2019; Yin et al., 2019). In the case of free amino acids, the content of most of them was increased by one or more Se dosages applied. Phenylalanine, in particular, was increased in both rocket species treated with high Se dosage; this amino acid is a substrate for aromatic GLS, but we did not identify any GLS derived from phenylalanine, though their existence in rocket is documented (Bell and Wagstaff, 2014; Bell et al., 2015; Toledo-Martín et al., 2017). Possibly, phenylalanine was preferentially used for the synthesis of phenols, as the enzyme phenylalanine ammonia-lyase (PAL) that uses this amino acid as a substrate can be induced by Se (Astaneh et al., 2018). A special note should be made for proline, because in the previous hydroponic study its content was substantially increased by Se in *D. tenuifolia*, while decreased in *E. sativa*. In the present study, however, we obtained the opposite result. Because proline acts as an osmolyte in cells to counteract osmotic stress, it is possible that its increase in *E. sativa* contributed along with other major osmolytes (e.g., non-structural sugars) to the plant's need to alleviate the osmotic imbalance due to the higher accumulation of Se in its tissues. In addition, proline exerts a protective effect on phospholipids, plasmalemma, mitochondria and plastid membranes (Naliwajski and Skłodowska, 2021), and it can contribute to the scavenging of hydroxyl radicals via a proline cycle (Signorelli et al., 2014). This cycle could also be coupled to the pentose phosphate pathway that generates erithrose-4-phosphate, a precursor of the shikimate pathway that leads to the production of chorismate, which is the branch point between primary and secondary metabolism and can promote the synthesis of secondary metabolites, including phenols (Shetty and Wahlqvist, 2004). Previously, a significantly more negative osmotic potential was found when *Brassica juncea* plants were treated with Se (unpublished data).

With respect to the phenolic compounds, various studies report contrasting results concerning the effect of Se on their synthesis and accumulation, which can depend on the form of Se applied to plants, the plant species and/or the method of Se administration (Robbins et al., 2005; Tian et al., 2016; D'Amato et al., 2018, 2020; Dall'Acqua et al., 2019). In our study, the effect of Se foliar administration on phenolic compounds had different effects in the two rocket species. Similar to the early hydroponic study, the net content of total phenolics in *E. sativa* was almost unchanged, although the level of some individual compounds increased and others decreased. In *D. tenuifolia*, the content of most phenolic compounds tested increased with Se supplementation, especially when plants were treated with the low Se dosage. This trend is opposite compared to those described for plants supplied with Se in hydroponics, as observed in the case of Cys, GLS, and certain free amino acids.

Conclusion

In conclusion, this study highlights the relevance of the method of Se supplementation in shaping the responses of the same plant species in terms of Se enrichment, S assimilation, synthesis, and accumulation of primary (e.g., amino acids, GSH) and secondary metabolites (GLS, phenolic compounds). Our results indicate that a single method of Se administration may not be the most suitable for a given plant species because the responses to different Se treatments could be very different, sometimes opposite. Therefore, before starting a large-scale biofortification program it would be more appropriate to carry out preliminary small-scale trials using the species to be enriched with Se to identify the most suitable method for applying Se. This must be done with the aim to biofortify plants with Se without compromising the content of other nutritionally valuable phytochemicals in the edible products.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MS: investigation, methodology, conceptualization, data curation, writing original draft, review and editing, and funding acquisition. EP-S: investigation, methodology, and review and editing. SN: review and editing and funding acquisition. SD: methodology, data curation, and review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

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Selenium enhances chilling stress tolerance in coffee species by modulating nutrient, carbohydrates, and amino acids content

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The effects of selenium (Se) on plant metabolism have been reported in several studies triggering plant tolerance to abiotic stresses, yet, the effects of Se on coffee plants under chilling stress are unclear. This study aimed to evaluate the effects of foliar Se application on coffee seedlings submitted to chilling stress and subsequent plant recovery. Two *Coffea* species, *Coffea arabica* cv. Arara, and *Coffea canephora* clone 31, were submitted to foliar application of sodium selenate solution (0.4 mg plant^{-1}) or a control foliar solution, then on day 2 plants were submitted to low temperature (10°C day/4°C night) for 2 days. After that, the temperature was restored to optimal (25°C day/20°C night) for 2 days. Leaf samples were collected three times (before, during, and after the chilling stress) to perform analyses. After the chilling stress, visual leaf injury was observed in both species; however, the damage was twofold higher in *C. canephora*. The lower effect of cold on *C. arabica* was correlated to the increase in ascorbate peroxidase and higher content of starch, sucrose, and total soluble sugars compared with *C. canephora,* as well as a reduction in reducing sugars and proline content during the stress and rewarming. Se increased the nitrogen and sulfur content before stress but reduced their content during low temperature. The reduced content of nitrogen and sulfur during stress indicates that they were remobilized to stem and roots. Se supply reduced the damage in *C. canephora* leaves by 24% compared with the control. However, there was no evidence of the Se effects on antioxidant enzymatic pathways or ROS activity during stress as previously reported in the literature. Se increased the content of catalase

during the rewarming. Se foliar supply also increased starch, amino acids, and proline, which may have reduced symptom expression in *C. canephora in* response to low temperature. In conclusion, Se foliar application can be used as a strategy to improve coffee tolerance under low-temperature changing nutrient remobilization, carbohydrate metabolism, and catalase activity in response to rewarming stress, but *C. arabica* and *C. canephora* respond differently to chilling stress and Se supply.

KEYWORDS

environmental changes, beneficial elements, abiotic stress, low temperature, tropical agriculture, plant nutrition, coffee belt

Introduction

Coffee is one of the most important commodities worldwide with a significant economic impact on over 25 million mostly smallholder farmers in more than 60 countries throughout the tropics (Jayakumar et al., 2017). Coffee plants are highly sensitive to the growing environment and are generally restricted to the "Coffee Belt"—between 25 degrees North and 30 degrees South with an average temperature between 18 and 22°C for *Coffea arabica* and 22 and 28°C for *Coffea canephora* (DaMatta and Ramalho, 2006; Descroix and Snoeck, 2004; Bunn et al., 2015; Bliss, 2017). Among the 104 *Coffea* species described (Davis and Rakotonasolo, 2008), the two most economically important species are *C. arabica* (Arabica) and *C. canephora* (Robusta). These two species are responsible for 99% of the world's green-bean production (Jayakumar et al., 2017).

Changes in the temperature due to climate change might adversely affect coffee plants because each species and genotype requires specific environmental conditions for successful production (Ramalho et al., 2014; Ebisa, 2017). Low-temperature stress may be denominated as (i) cold stress—when plants suffer from sub-zero temperatures, and (ii) chilling stress—when plants suffer from low but non-freezing temperatures (Graves, 1995). As a result of chilling stress, plants have shown reduced stomatal conductance, changes in the pigment complexes and losses of photochemical efficiency, restricted electron transportation, and changes in carbon metabolism, allocation, and partitioning (Ensminger et al., 2006; Partelli et al., 2010; Batista-Santos et al., 2011).

Acclimation to low temperature is usually initiated by a short-term fluctuation in temperature, which affects metabolic homeostasis and induces a stress response (Ensminger et al., 2006). A sudden drop in temperature limits the ability of plants to induce protective metabolic responses. Severe frosts in 2021 were experienced in coffee areas in the southeast of Brazil, the highest production region of Brazil, with almost 8–10% of the arabica coffee affected, which reduced the production in the order of 17% below recent on-year crops (Usda Foreign Agricultural Service, and United States, 2022). Exogenous application of beneficial elements, such as selenium (Se), has emerged as a tool to compensate for the negative impacts of many stresses, including chilling (Brown et al., 2021; Zellner et al., 2021).

Although Se is not an essential element for higher plants, it has been shown to increase antioxidant activity (Ekanayake et al., 2015), change carbohydrate metabolism (Lara et al., 2019; Silva et al., 2020), protect chlorophyll, and modulate water relations (Zhang et al., 2014). Se application has reduced the side effects of abiotic stress in a wide range of staple crops, such as drought in common beans and rice (Andrade et al., 2018; Ravello et al., 2021), heavy metal exposure in wheat (Liu et al., 2021; Hasanuzzaman et al., 2022), and salinity in maize and garlic (Ashraf et al., 2018; Astaneh et al., 2019).

Previous studies resulted in higher coffee yield in response to Se supply by increasing antioxidant metabolism (de Mateus et al., 2021); however, there have been no studies that explore the influence of Se application in coffee species under chilling stress. Here, the effects of Se supply to coffee plants under chilling on plant metabolic responses and plant tolerance were examined.

Materials and methods

Plant material

The trial was performed using two different coffee species, *C. arabica* cv. Arara and *C. canephora* clone 31, differing in tolerance to low temperature (DaMatta and Ramalho, 2006). According to these authors, low-temperature tolerance is related to the species' ability to change its metabolism to trigger adverse conditions (e.g., increases in enzymatic activities, lipids quantitative and qualitative changes, protection of proteins in cell membranes). The plants were provided by the National Institute of Science and Technology of

Coffee (INCT *Café*). Plants with 5–6 pairs of fully expanded leaves were used. They were selected for high health and uniformity, and allowed to acclimate under optimal conditions for 14 days in a Conviron® growth chamber [12 h of photoperiod, 60% relative humidity (RH), 260 μmol m^{-2} s^{-1} of light intensity (during the day), and optimal temperature (25°C_{day}/20°C_{night})]. Coffee seedlings were grown on 1 l of a substrate composed of subsoil + cattle manure at a ratio of 3:1, with 5 g of single superphosphate being added to each kilogram of the mixture. The irrigation was made dairy with 80 mL of deionized water during the optimal temperature and 15 mL of deionized water during the chilling temperature.

Experimental design and treatments

The experiment was arranged in a randomized block design and a 2 × 2 factorial scheme with five replicas of seedlings for each treatment, with the experimental unit consisting of three pots totaling 60 pots. The factorial scheme was composed of two species (*C. arabica* cv. Arara and *C. canephora* clone 31), in the absence and presence of Se (0 and 80 mg L^{-1} Se). Samples were collected three times to evaluate plant responses before, during, and after exposure to chilling stress. Considering the great number of leaves that needed to be collected at each time of evaluation, each replication was composed of three seedlings. The Se rate used in the trial was based on preliminary testing (unpublished data) with coffee seedlings and also on results found for other crops. The control treatment is hereafter described as the plants of the respective species analyzed before being submitted to the chilling stress.

Fourteen days after being transferred to the growth chamber, the plants were moved to a spray chamber to avoid contamination during the foliar treatment application. Thus, the respective Se treatments plants were sprayed manually to drip with 5 mL of a foliar solution of Se (80 mg L^{-1} Se + 0.5% v/v of mineral oil) and the remaining plants were sprayed with mineral oil solution (0.5% v/v of mineral oil). Plants were then returned to the growth chamber. The Se source used was sodium selenate (Na_2SeO_4—Sigma Aldrich 98.9%).

The first foliar sampling was performed 7 days after the foliar treatment application. All plants were then exposed to chilling temperatures, which were decreased by 5°C/h from 25 to 10°C during the first day. The temperature was set to 4°C during the night and 10°C during the day (12 h of photoperiod, 60% RH, 260 μmol m^{-2} s^{-1} of light intensity). The temperature regime was defined as suboptimal for coffee growing (Ramalho et al., 2003; DaMatta and Ramalho, 2006).

The second foliar sampling was performed 2 days after low-temperature stress treatment. The temperature was then returned to optimal conditions (25°C_{day}/20°C_{night}), and the third sampling was performed 2 days later (post-stress).

Assessments

Visual damage scale

The visual damage from low-temperature exposure in the leaves was carried out according to Manetti Filho and Caramori (1986). The scale of damage ranged from 1 to 5, in this way: (1) no damage; (2) 0–25% of the total leaf area damaged; (3) 25–50% of the total leaf area damaged; (4) 50–75% of the total leaf area damaged; and (5) representing visual damages from 75 to 100% of the total leaf area. The visual damage scale from low-temperature exposure in the leaves was performed considering the general appearance of all leaves.

Sample collection and preparation

Two leaf samples were collected for different groups of analyses as follows: (1) The third and fourth fully expanded pairs of leaves from top to bottom of coffee plants were collected and washed three times with distilled water. Then, the samples were dried for 72 h at 60°C and ground in a Willey mill to obtain the dried leaf tissue. The dried samples were used to quantify the parameters described in section 2.3.3 (total content of Se, nitrogen, and sulfur), section 2.3.7 (carbohydrates, total protein, total free amino acids), and section 2.3.8 (proline); and (2) The second fully expanded pair of leaves from top to bottom of coffee leaves were collected 2 h after lights-on then immediately snap-frozen in liquid nitrogen, individually macerated in liquid nitrogen, homogenized in a cooled mortar using 100 mg PVPP (antioxidant), and stored at -80°C. The dried tissue was used to perform the analysis of the content of Se, sulfur, nitrogen, carbohydrates, total protein, total free amino acids, and proline. The frozen tissue was used to quantify the parameters described in sections "Calculation of LOD, LOQ, and reference material recovery" (antioxidant enzymes) and "Antioxidant enzymes (**s**uperoxide dismutase, catalase, **a**scorbate peroxidase, and glutathione reductase)" (hydrogen peroxide and lipid peroxidation).

The sample collection was repeated before, during, and after chilling stress. Since this procedure is a destructive analysis, one plant of the experimental unit was used in each sample collection.

Total content of selenium, sulfur, and nitrogen

The extracts for the quantification of Se and S in leaves were obtained by acid digestion of 0.5 g of the dried sample according to the USEPA 3051A protocol (USEPA, 2007) in a microwave (Mars 5, CEM Corporation, Matthews, NC, United States). A blank and certified reference material for Se (White clover, BCR402-IRMM) was included in each batch of samples. The Se content in the leaves was measured using GFAAS (Graphite Furnace Atomic Absorption Spectrometry, Atomic Absorption Spectrometry with Zeeman background correction and EDL lamp for Se; Analyst™ 800 AAS, Perkin Elmer), and the S content was measured using ICP-OES (Inductive Coupled

Plasma Emission Spectrometry, Spectro, Blue model, Germany). Total N contents were determined by sulfur digestion and Kjeldahl distillation (Tecnal, TE-136, Brazil) (Malavolta et al., 1997).

Calculation of LOD, LOQ, and reference material recovery

The detection and quantification limits (LOD and LOQ) were calculated with three and 10 times the standard deviation (LOD and LOQ, respectively) of 10 individually prepared blank solutions (Silva Junior et al., 2017). The LOD and LOQ for Se were, respectively, 4.26 and 12.2 $\mu g\ kg^{-1}$. The Se recovery rate in the reference material was 95.2% ± 4.1.

Antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase)

Frozen leaf tissue was weighed (0.2 g) and mixed with 1.5 mL of potassium phosphate buffer solution (0.1 mol L^{-1}, pH 7.8 + 0.1 mol L^{-1} EDTA, pH 7.0, 0.01 mol L^{-1} ascorbic acid, and 22 mg polyvinylpolypyrrolidone-PVPP). The suspension was centrifuged at 14,000 *g* for 10 min at 4°C (Biemelt et al., 1998). The supernatant was used to assess the activity of the antioxidant enzymes. Quality assurance and quality control of the enzymatic analyses were warranted by using two blanks in each reading plate and operating the samples at 0–4°C. In addition, the enzyme extraction was performed on the day of the analysis to avoid the oxidation of the enzyme extract.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium at 560 nm (Giannopolitis and Ries, 1977). The reading sample was composed of 50 mM of potassium phosphate buffer, pH 7.8, 14 mM methionine, 0.1 μM EDTA, 75 μM NBT, 2 μL of enzyme extract, and 2 μM riboflavin.

Catalase (CAT, EC:1.11.1.6) activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm (Havir and McHale, 1987). For this, were pipetted 100 mM of buffer solution of potassium phosphate 200 mM pH 7.0, 12.5 mM H_2O_2, and 3 μL of enzyme extract. The CAT activity was read every 15 s for 3 min and was defined as the amount of enzyme necessary to reduce 1 $\mu mol\ H_2O_2\ min^{-1}$.

Ascorbate peroxidase (APX, EC:1.11.1.11) was determined by the method of reduction of ascorbate at 290 nm (Nakano and Asada, 1981). In order to quantify the APX, 50 mM potassium phosphate 100 mM pH 6.0, 0.8 mM ascorbic acid, 1 mM H_2O_2, and 3 μL of the enzyme. The APX activity was read every 15 s for 3 min and was defined as the amount of the enzyme required to oxidize 1 mmol (ascorbate) min^{-1}.

Glutathione reductase (GR, EC:1.6.4.2) was assayed according to the methodology proposed by Schaedle and Bassham (1977) and adapted by García-Limones et al. (2002). The GR activity was read at 340 nm. The reaction medium consisted of 50 mM of buffer solution of potassium phosphate pH 7.8, 0.5 mM oxidized glutathione, 3.0 mM $MgCl_2$,0.15 mM NADPH, and 15 μL of enzyme extract. One GR unit is defined as the amount of enzyme that oxidizes 1 $mmol\ min^{-1}$ NADPH.

The analyses were carried out in triplicates and were measured using an Epoch® Microplate Spectrophotometer (BioTek, United States).

Hydrogen peroxide and lipid peroxidation (malonaldehyde)

Frozen leaf tissue (0.2 g) was ground in liquid nitrogen, homogenized in 5 mL of trichloroacetic acid (TCA), and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was collected to determine hydrogen peroxide (Velikova et al., 2000) with adaptations of Loreto and Velikova (2001). Lipid peroxidation (MDA) was assayed according to Buege and Aust (1978) and Silva et al. (2020).

For the determination of hydrogen peroxide, 0.45 mL of supernatant were added to 2.5 mM potassium phosphate buffer, pH 7.0, and 0.5 mM potassium iodate. The absorbance of the supernatant was read at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve previously made using different concentrations of H_2O_2.

The assay of lipid peroxidation (MDA) was carried out by the thiobarbituric acid (TBA) test, which determines the MDA as an end product of lipid peroxidation. Then, 0.125 mL of the supernatant was added to 0.25 mL of a mixed solution of TBA (0.5%) and TCA (10%). The mixture was incubated in a water bath at 95°C for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The absorbance of the supernatant was measured at 532 nm, subtracting the value for non-specific absorption at 600 nm. This procedure was made in duplicates.

Carbohydrates, total protein, and total free amino acids

The extraction of carbohydrates and proteins was based on Zanandrea et al. (2010). Individual dried leaf samples were weighed (0.2 g), mixed with 5 mL of potassium phosphate buffer (pH 7.0), and heated in a water bath at 30°C for 40 min. Then, the suspension was centrifuged at 10,000 *g* for 20 min and the supernatant was collected. This procedure was done twice and both supernatants were mixed. The same pellet was used for starch extraction mixing 8 mL of potassium acetate buffer (200 mM pH 4.8) and 2 mL of amyloglucosidase (1 $mg\ mL^{-1}$; 16 units of enzyme). Then, the samples were heated in a water bath at 40°C for 120 min and centrifuged for 20 min at 10,000 *g*. The supernatants were collected for measurements. The contents of starch, sucrose (Suc), and total soluble sugars (TSS) were determined using the anthrone method (Dische, 1962). Reducing sugars were determined according to the DNS method (Miller, 1959), and total free amino acids (AA) were determined according to the ninhydrin

method (Yemm et al., 1955). The protein content (Prt) in the leaves was also determined (Bradford, 1976).

Proline

Proline content was assessed by the colorimetric method originally described by Bates et al. (1973) with minor modifications. The dried leaf tissue (0.1 g) was weighed and macerated with sulfosalicylic acid 3%. Next, samples were mixed for 60 min at environmental temperature. After the extraction, the content of Pro in the leaves was determined by adding 0.5 mL of extract, 1.5 mL of deionized water, 2 mL of a freshly prepared acid-ninhydrin solution, and 2 mL of pure acetic acid. Tubes were incubated in a water bath at 100°C for 60 min. The reaction was stopped by placing the reaction tubes in an ice bath. The supernatant was carefully collected and read at 520 nm.

Statistical analysis

The statistical analyses were performed using the R software (R Core Team, 2021). An exploratory analysis of data was first performed to verify the existence of outliers. Then, the analysis of variance (ANOVA) was conducted on the data after the validation of the model and tests of assumptions (normality, homoscedasticity, independence, and additivity of residuals). When significative ($p < 0.05$), the interaction of the studied factors (Se supply and coffee genotypes) was compared. When there was no interaction between tested factors ($p > 0.05$), the means of the treatments were compared at each factor. Means were compared using the Tukey test ($p < 0.05$). In addition, principal component analyses (PCAs) were performed to determine the relationships of the measured variables. Pearson's correlation analysis ($p < 0.05$) was performed to validate the relationships observed in PCA. PCA and correlation analysis was performed for each species and time of evaluation (before, during, and after stress). The correlation matrices among variables are reported in **Supplementary Material**.

Results

Visual damage scale

Leaf visual damage was influenced by species and Se supply (**Figure 1**). *C. canephora* was statistically ($p < 0.05$) more affected than *C. arabica* at both evaluation times. During the stress, the damage to *C. canephora* was twofold higher than in *C. arabica* (**Figure 1A**). Se supply reduced the damage by low temperature in *C. canephora* by 24 and 17% compared with its initial control value at optimal temperature (25°C day/20°C night), respectively, for the evaluations performed during chilling and the rewarming (**Figures 1A,B**).

C. canephora showed main leaf damage in the leaves with a yellowish-green color during and after the cold stress (**Figure 1C**). Although the *C. arabica* did not show high damage by cold, slight darkened damage in the leaves after 2 days of exposure to chilling stress was observed (**Figure 1C**).

Analysis of selenium, sulfur, and nitrogen

Leaf Se content ranged from 0.18 mg kg^{-1} DW (control treatment) to 2.13 mg kg^{-1} DW (after chilling stress) in the *C. arabica* and 0.18 mg kg^{-1} DW (control treatment) to 1.81 mg kg^{-1} DW (after chilling stress) in the *C. canephora*. There was no statistical difference between the species (**Figure 2**).

In this study, Se foliar supply increased the N content in the leaves before plants were submitted to chilling stress, but N content was reduced in the low-temperature condition by Se application (**Figure 2**).

The leaf S content was affected by species and Se supply in all the evaluation times ($p < 0.05$). The S content in *C. canephora* was significantly higher than in *C. arabica*. Se foliar supply promoted 9% higher S content in leaves on the evaluation performed before the cold, but Se supply reduced the S content in the leaves during and after stress (**Figure 2**). The S content decreased 10.5 and 10.7%, respectively, during and after chilling stress by Se application.

Antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase)

The average values of antioxidant enzyme activity (GR, SOD, CAT, and APX), as well as the hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA), assessed in the species treated and non-treated with foliar Se are presented in **Figure 3** and **Supplementary Table 1**.

Chilling stress promoted an increase of 44% in GR activity in non-treated plants with Se, but these plants were unable to keep high GR activity during the rewarming condition and the GR activity was reduced by 97% (**Figure 3C** and **Supplementary Table 1**). On the other hand, the Se supply was responsible for statically increasing the GR after the chilling stress compared with non-treated plants with Se.

The SOD activity was notably increased during chilling stress compared with optimal temperature conditions. After stress, SOD was affected by the interaction of the two factors (Species × Se supply). Foliar supply promoted 23.5% higher SOD activity in *C. arabica* (**Figure 3D** and **Supplementary Table 1**). The same effect was not shown in the *C. canephora*.

Foliar supply of Se promoted 50% less CAT activity in *C. canephora* than the same non-treated species during chilling

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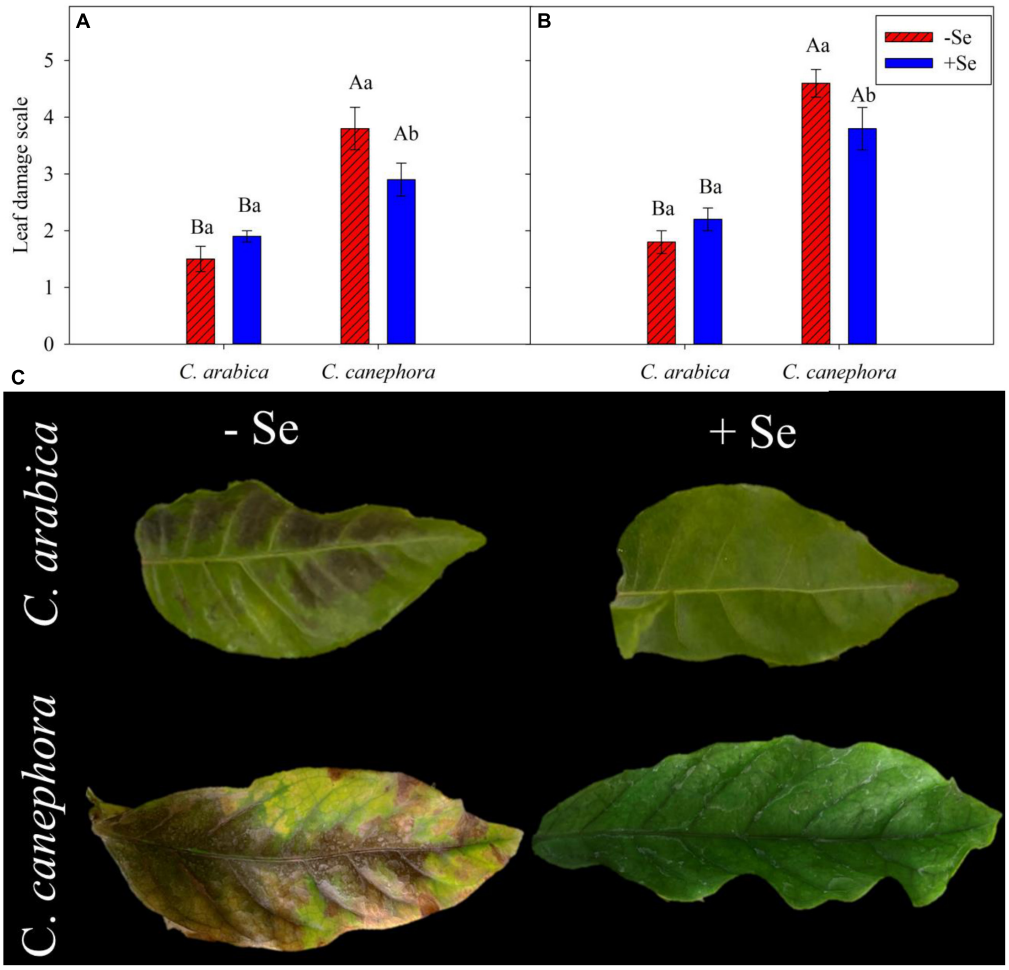

FIGURE 1
Leaf visual damage in coffee species exposed to chilling stress and two conditions of Se foliar supply after 2 days of exposure to low temperature. Visual damage scale during **(A)** and after stress **(B)** according to Manetti Filho and Caramori (1986). **(C)** Visual damage in coffee after low-temperature stress. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype and different uppercase letters indicate significant differences within each genotype (*C. arabica* and *C. canephora*) in each Se supply condition are significantly different ($p < 0.05$, $n = 5$) by Tukey multiple comparison test. Vertical bars represent the standard error.

stress. Moreover, Se foliar supply increased CAT activity before and after the stress, independently of the species ($p < 0.05$). APX activity was not influenced by Se application and was affected by the species in which *C. arabica* showed higher activity regardless during and after chilling (**Figure 3A** and **Supplementary Table 1**).

Hydrogen peroxide and lipid peroxidation (malonaldehyde)

Levels of malonaldehyde (MDA) and H_2O_2 were not influenced by the presence of Se and species (**Figures 3E,F** and **Supplementary Table 1**). The stress increased the MDA content by 7.7 and 35.6% in the *C. arabica* and *C. canephora* compared with the respective genotype before the stress. After the stress, MDA increased by 58.3 and 38%, respectively, for *C. arabica* and *C. canephora* compared with the same species before stress. This supports the hypothesis that *C. canephora* has less ability to tolerate low temperatures than *C. arabica* because the MDA content increased promptly after the plants were submitted to chilling stress. On the other hand, MDA content in the *C. arabica* showed subtle adjustment during the stress but increased abruptly from 36.2 to 53.2 nmol g^{-1} FW^{-1} during the rewarming.

Carbohydrates, total protein, and amino acids

The Suc content in leaves was affected by the species in all periods of evaluation and *C. arabica* had higher Suc content than

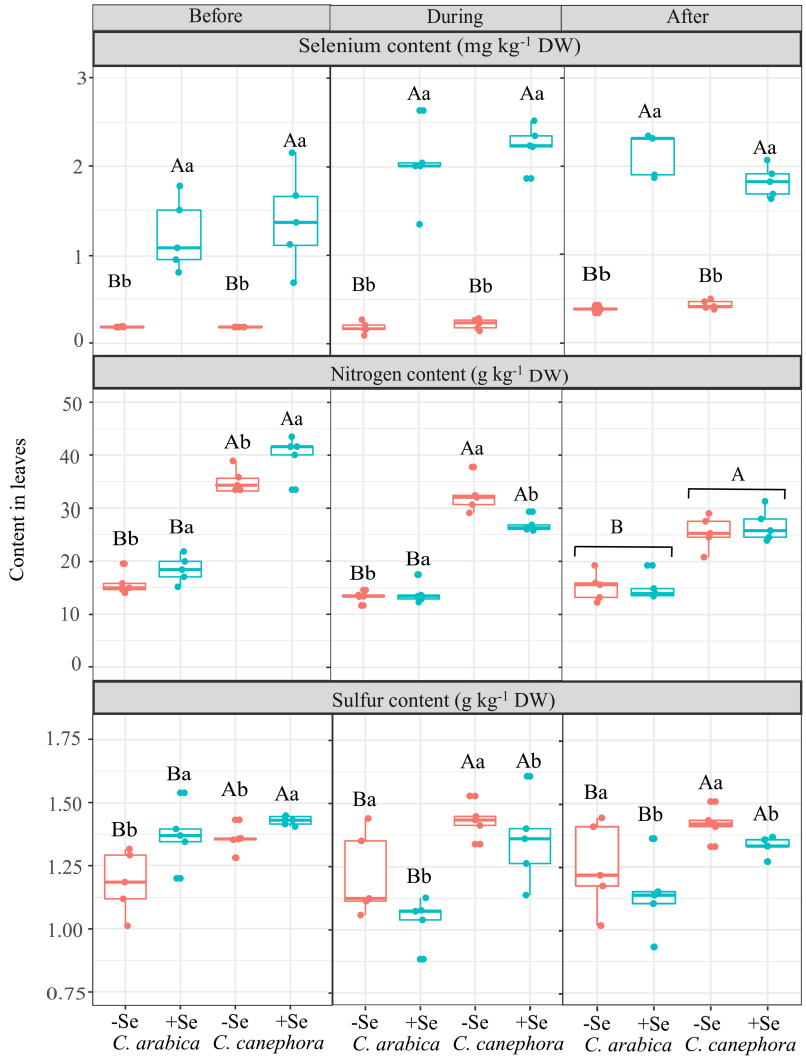

FIGURE 2
Effects of Se foliar application and temperature condition on Se, S, and N content in leaves of *C. arabica* and *C. canephora*. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype are significantly different ($p < 0.05$, $n = 5$) by Tukey multiple comparison tests as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*). Vertical bars represent the standard error.

C. canephora. In addition, *C. arabica* showed less impact from chilling stress on Suc (**Supplementary Table 2**).

C. canephora showed less ability to maintain the initial content of Suc and RS after exposure to low temperature than *C. arabica*. The reduction of Suc and RS in the *C. canephora* was 22.7 and 25.7%, respectively. During rewarming, the *C. canephora* plants were unable to increase the Suc and RS content as *C. arabica*, showing a reduction of 45.2 and 44.2% compared with the plants before the stress. The *C. arabica* plants also showed a subtle reduction in Suc when exposed to chilling stress, but it was less pronounced than in *C. canephora*. Meanwhile, the *C. arabica* plants reduced the RS content in the leaves during the stress, but its content was increased by 8.8% in the rewarming period.

The Se foliar application promoted lower starch content in the plants before and during stress, but its supply modulated the starch content after the plants were subjected to chilling stress, which led to an increase of ~30.7% in the starch when compared with plants that did not receive Se foliar application (**Figure 4A**). In addition, Pearson's correlation analysis showed a positive correlation ($p < 0.05$) of Se and starch in both species after the chilling stress—$R^2 = 0.92$ and $R^2 = 0.68$, respectively, to *C. arabica* and *C. canephora* (**Supplementary Figures 5, 6**). There were significant differences ($p < 0.05$) between Se supply in the TSS content before and after the stress. The TSS content in foliar tissue from Se-supplied plants was 18% lower than in those that did not receive Se supply (**Figure 4**). Foliar supply reduced the TSS content before stress. In contrast, the Se supply

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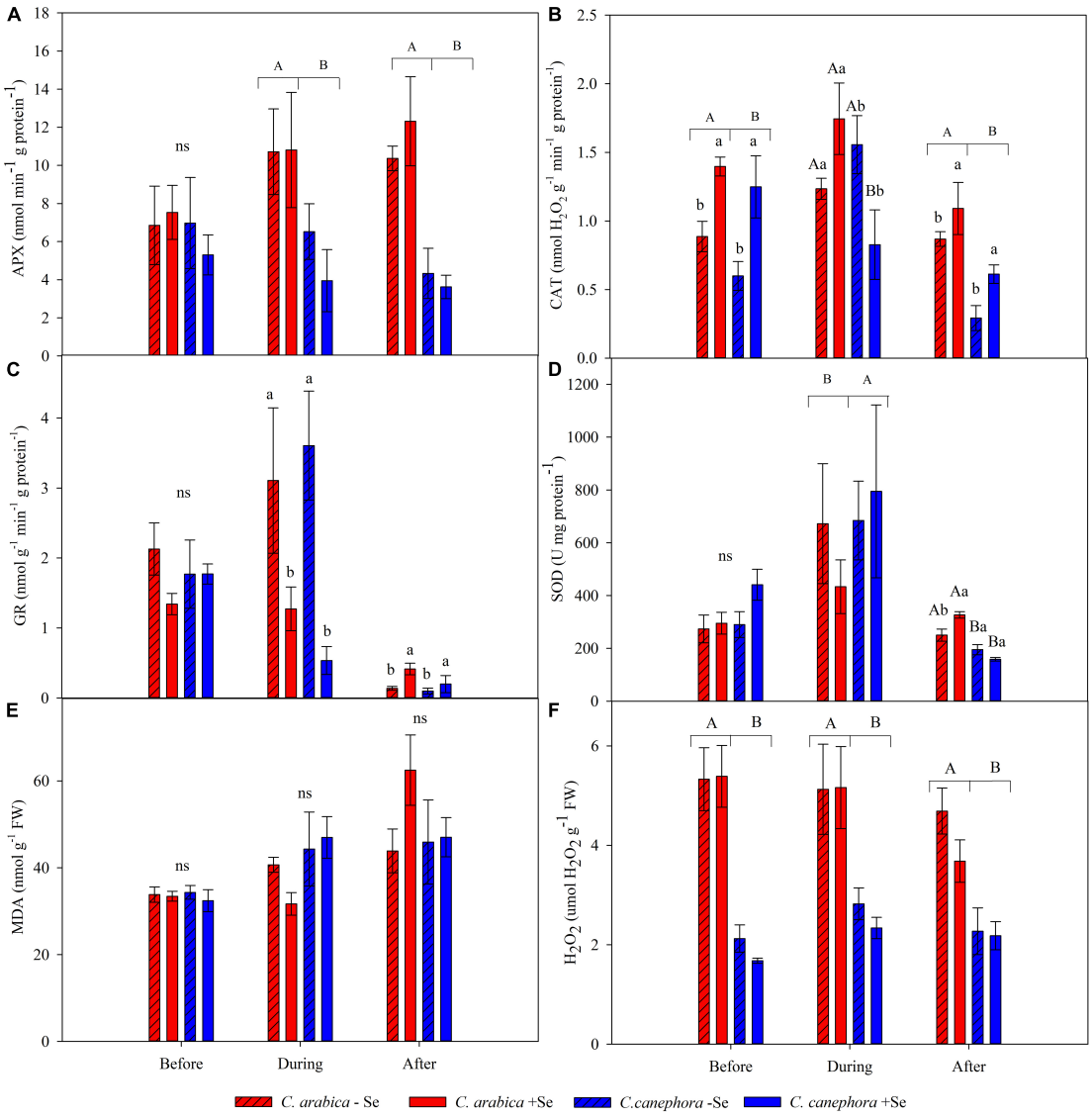

FIGURE 3
Effects of Se foliar application on APX **(A)**, CAT **(B)**, GR **(C)**, SOD **(D)**, MDA **(E)**, and H_2O_2 **(F)** in leaves of *C. arabica* and *C. canephora* during the rewarming. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype are significantly different ($p < 0.05$, $n = 5$) by Tukey multiple comparison tests as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*). Vertical bars represent the standard error.

increased the TSS content after the chilling stress in both species. After the stress, TSS showed a correlation with Se content in the leaves according to PCAs (**Figures 4E,F**). This behavior is also supported by a significant correlation ($p < 0.05$) to Se content in leaves in both species according to Pearson's correlation analysis (**Supplementary Figures 5**, **6**).

The application of Se improved the Prt content in *C. arabica* leaves before the plants were submitted to chilling stress, but this effect was not noticed during the chilling stress and the rewarming period. Despite this, Prt was higher in *C. canephora* than in *C. arabica* during all growth temperature conditions. Similarly, the AA content was higher in *C. canephora* than in *C. arabica*, where the AA content was not influenced by Se application before and after the chilling stress.

Proline

The Pro content was affected by species before and during chilling stress, in which *C. canephora* has shown notably higher content than *C. arabica*. Nevertheless, Pro content in *C. canephora* during the stress was reduced by 44 % after the stress, showing that the low temperature can exert great influence on the Pro content in stress conditions. Despite the

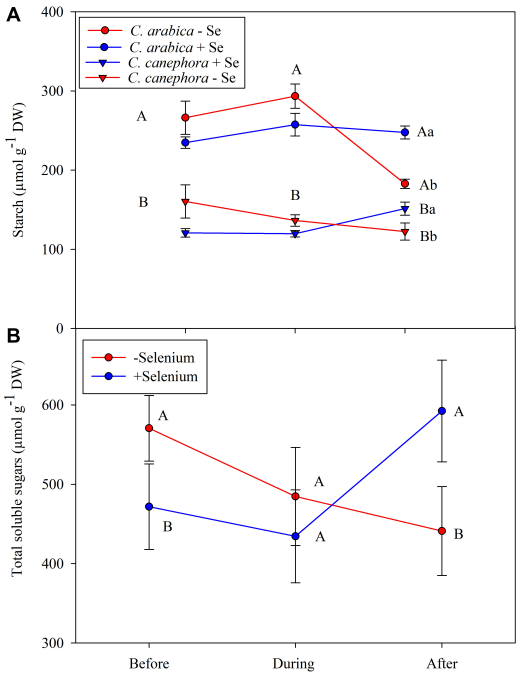

FIGURE 4
Effects of Se foliar application and temperature conditions on Starch **(A)** and TSS **(B)** content in leaves of *C. arabica* and *C. canephora*. The TSS content was obtained by the average of both species. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype are significantly different ($p < 0.05$, $n = 5$) by Tukey multiple comparison tests as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*). Vertical bars represent the standard error.

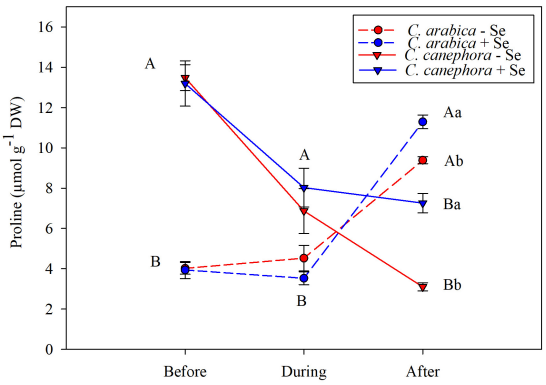

FIGURE 5
Effects of Se foliar application and temperature conditions on Pro content in leaves of *C. arabica* and *C. canephora* during the rewarming. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype are significantly different ($p < 0.05$, $n = 5$) by Tukey multiple comparison tests as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*). Vertical bars represent the standard error.

lower initial Pro content in the *C. arabica*, this genotype was able to increase significantly the content in the rewarming, which was potentialized by the Se application. Se application increased 20.4 and 133% of the Pro content, respectively, to *C. arabica* and *C. canephora* without Se application (**Figure 5**).

Principal component analysis

The principal component analysis (PCA) showed that the relations between the analyzed parameters and Se content in leaves vary as a function of species and temperature conditions (before, during, and after chilling stress). Overall, it is possible to find two groups enclosed in the ellipses, which are composed of samples supplied with Se at all evaluation times (**Figure 6**).

Before stress in *C. arabica*, the application of Se promoted the higher contents of Se, and this Se had a positive relationship with CAT and the content of S and a negative relation with AA (**Figure 6**). During stress, the positive relationship between Se content and CAT was maintained, with CAT having also a positive correlation with Prt. After chilling stress, the relationship between CAT and Se content was not maintained. Se content was increased by its application and had a positive correlation with Pro, TSS, Sta, GR, and SOD.

For *C. canephora* after chilling, the PCA showed a positive and significant correlation ($p < 0.05$) of Se with CAT, Sta, Suc, TSS, and Pro, which was supported by the correlation matrix (**Supplementary Figure 6**).

A negative relationship was observed between S and TSS content. During the low-temperature stress, the Se content showed a negative relationship with the content of N, AA, Sta, CAT, and GR. After the stress in *C. canephora,* Se content had a positive relationship with Pro, TSS, Sta, Suc, and CAT.

Discussion

The stress promoted by chilling impacted negatively plant development and caused significant damage to the leaves. Plant exposure to low-temperature stress commonly reduces the physiological parameters (e.g., stomatal conductance, photosynthetic rate, and intercellular CO_2 concentration) (Huang et al., 2018). Stomatal closure has been related as one of the first plant mechanism responses to cold stress, conferring stress tolerance during low-temperature stress and the reduction of CO_2. Low CO_2 fixation can reduce the photosynthetic rate by causing disequilibrium between light capturing and utilization, as well as by changing the photochemistry of chloroplasts. The excess light energy in the photosystems causes an imbalance between electron release and acceptance, which increases ROS formation (Larcher, 1985; de Oliveira et al., 2002).

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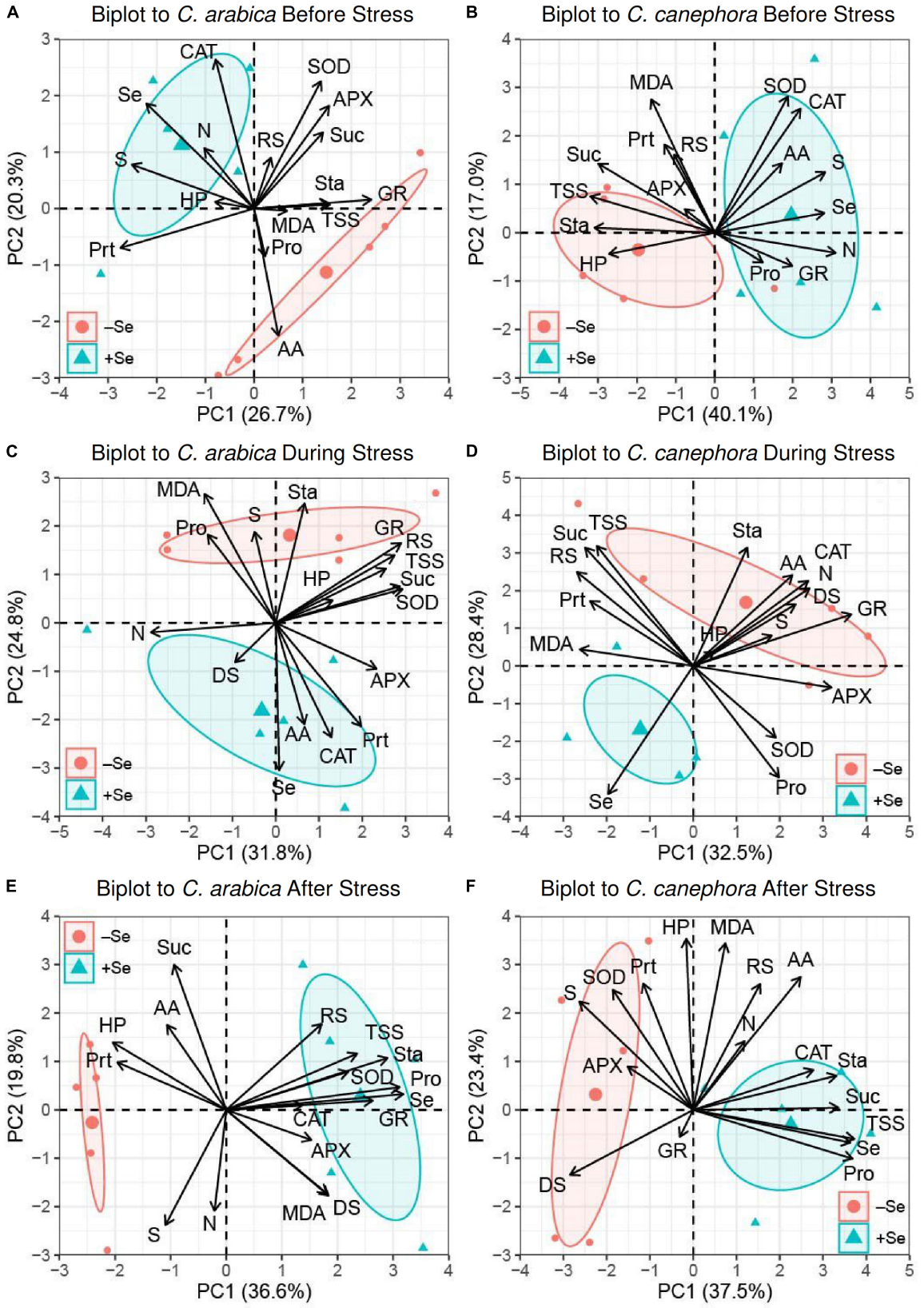

FIGURE 6
PCAs biplot representation of leaves composition data before, during, and after chilling stress in two species. **(A,C,E)** *C. arabica* before, during, and after stress, respectively; **(B,D,F)** *C. canephora* before, during, and after stress, respectively.

Partelli et al. (2009) showed that increased ROS promotes lipidic peroxidation and loss of membrane selectivity, then, coffee plants submitted to low temperature have shown chlorophyll loss and leaf tissue degradation, reflecting injuries in the leaves. These leaf damages were also observed in this trial (**Figure 1**). According to Huang et al. (2018), the foliar supply of Se reduced significantly the foliar damage of chilling stress in strawberries, and the authors conferred this behavior to the enhanced gas exchange during the stress. Their results also showed that Se alleviated chlorophyll degradation and reduced MDA and H_2O_2. Additionally, it has been shown that Se application increases the stability of the photosynthetic machinery, while also preserving the membrane system, which promotes higher tolerance to low temperatures (Yang et al., 2021).

The higher damage in the *C. canephora* leaves compared with *C. arabica* suggests that each genotype might act distinctly when submitted to stress in the triggering of metabolic responses to temperature changes (Petek et al., 2005; Fortunato et al., 2010; DaMatta et al., 2018), including different responses to Se application. These results are explained by the allopolyploidy of *C. arabica*, which promotes an evolutionary advantage in having additional genetic materials that attribute greater plasticity in coping with environmental variations compared with its parentals—in this case, *C. canephora* and *C. eugenioides*. In other words, the allopolyploidy of *C. arabica* makes this species able to up and downregulate certain genes responsible to keep the homeostasis during low temperatures, as reported by Bardil et al. (2011), or even at higher temperatures (de Oliveira et al., 2020).

The increase in the S content in the coffee leaves before the chilling stress probably occurred due to its intimate relation with Se metabolism in plants. Currently, some studies have shown that the high-affinity sulfate transporters involved in sulfate uptake and translocation throughout plant tissues may be utilized by selenate ($NaSeO_4$) as well (Sors et al., 2005; White, 2018). At this point, low content of Se can improve S uptake by mimicking S deficiency to activate specific sulfate transporter expression and stimulate S uptake, resulting in the selenate-induced S accumulation (Boldrin et al., 2016).

The higher S content in leaves led to an increase in the Prt before stress, which was supported by significative Pearson's correlation ($R^2 = 0.80$, $p < 0.05$) in the *C. arabica* (**Supplementary Figure 1**). Sulfur is a structural constituent of certain amino acids (e.g., methionine; Met and cysteine; Cys) and coenzymes, as well as in prosthetic groups such as ferredoxin, essential composts for plants to survive in unfavorable conditions (Saleem et al., 2021).

In addition, S composes the amylase molecule through Cys. Since Cys compose the amylase, this amino acid can increase amylase activity aiming to face the stress. Then, most of the stored source of carbohydrates is degraded by amylase and the product is then supplied to the plants for energy and carbon for growth (Thalmann and Santelia, 2017). Meanwhile, Se supply can also stimulate amylase by the same mechanisms endorsed by S, since they share the same primary metabolism in plants and Se can be incorporated in Cys, giving rise to Se-Cys (Jacob et al., 2003; White, 2018).

The reduction of S content during and after chilling stress by Se supply could be connected with the potential changes in energetic metabolism of plants under severe stress, which cause its remobilization from leaves to storage parts, such as roots and stems. The storage of nutrients may be an effective alternative for sustaining plant growth and plays a key role in energy-saving during the rewarming condition (Etienne et al., 2018).

Lipid peroxidation is a good indicator of ROS activity on cell damage, mainly because oxidative stress causes the peroxidation of unsaturated fatty acids, whereas increasing MDA concentration (Farooq et al., 2019). Exogenous Se supply has also been related to reduced ROS, such as H_2O_2, and lipidic peroxidation under stress conditions (Jóźwiak and Politycka, 2019; Silva et al., 2020; de Mateus et al., 2021). However, this behavior was not seen in this trial with coffee (**Supplementary Table 1**). Despite this, the Se supply significantly reduced injuries to the leaf tissue of *C. canephora* plants during and after the stress (**Figure 1**), suggesting that the negative effects of chilling stress are mitigated by pathways other than ROS scavenging.

The effect of Se on the improvement of antioxidant enzyme activity during chilling stress has been reported elsewhere (Chu et al., 2010; Abbas, 2012). In fact, plants can acclimate—i.e., can adjust to changes in their environment— to some extent, sustaining effective metabolism as a result of a variety of complimentary mechanisms that defend the cell. However, under extreme conditions plants may not be able to adapt to environmental disturbances, which results in severe damage to cell structures and also to proteins involved in the physiological metabolism (DaMatta et al., 2018). In addition, antioxidant enzymes are highly dependent on protein functions and low temperatures can lead the proteins to reduce their activity and lower cellular fluidity (Maksimov et al., 2017).

The higher content of Se in leaves and the remobilization of S from shoot to roots probably are correlated with de TSS, starch, AA, and Pro content during the rewarming (**Supplementary Table 2** and **Figure 4**). It can be suggested based on data that to keep the carbohydrate demand for growth under low temperature, Se can help plants to remobilize the S from leaves after the stress.

In this way, Se application helped the plants to maintain the starch content during the rewarming, since Se increased starch content by 12% compared with the same treatment before the chilling stress (**Figure 4** and **Supplementary Table 2**). On the other hand, control treatments showed a reduced starch content by 28%. These results show that foliar Se cannot only reduce the starch breakdown but also increase the content after the low-temperature stress compared with those that do not receive foliar Se.

Provided that the effect of chilling stress includes impairment of photosynthesis, Se supply in plants cause increases in the structure and functionality of the photosynthetic apparatus, allowing the plants to maintain higher net photosynthesis during stress condition (Lara et al., 2019; Souza et al., 2019). At this point, transitory starch is synthesized in the leaves directly from photosynthates during the day and can be degraded the following night to sustain metabolism, energy production, and biosynthesis in the absence of photosynthesis (Pfister and Zeeman, 2016). According to Stein and Granot (2019) and Ribeiro et al. (2022), starch not only acts in the energetic metabolism, but also as promoting rapid stomatal opening, making osmoprotectants, cryoprotectants, scavengers of free radicals and signals, and reverting embolized vessels. Besides, its cleavage products are available for many metabolic pathways, including the synthesis of complex carbohydrates.

According to PC1, during the rewarming, the effect of Se on Suc was positive in *C. canephora*, but negative in *C. arabica*. Moreover, Suc was found on the opposite side of DS in PCA1 (**Figure 6F**) and also significantly negative according to Pearson's correlation ($R^2 > 0.74$) (**Supplementary Figure 6**). In addition to higher Suc, the Se application also promoted higher TSS, total amino acids (AA), and Pro content in leaves, regardless of genotype during the rewarming (**Supplementary Table 2**). These results evidence that, although *C. canephora* plants were not able to maintain their full development during the stress, Se supply can impair plant metabolism after the low-temperature stress, which results in less damage to *C. canephora* plants.

Proline content was affected by the species before and during low-temperature stress and *C. canephora* showed higher content than *C. arabica*. Nevertheless, the *C. canephora* reduced the content of Pro by 43% when submitted to low temperature, and 60% during the rewarming. Meanwhile, the Pro content in *C. arabica* maintained the same status during chilling stress but increased by 15% compared with Pro content before stress. Although Se affected positively the Pro content in both species, it is remarkably in *C. canephora* (135%) when compared with *C. arabica* (20%).

The considerable depletion of Pro content in *C. canephora* showed that this specie had less ability to survive during the stress. In contrast, *C. arabica* was able to modulate the content of Pro to protect the cellular structures and reduce the production of ROS. It is also supported by the allopolyploidy of *C. arabica*, in which these plants are able to activate different genes to induce the production of Pro in the rewarming and downregulate its content in the *C. canephora*. Moreover, the regulation of these genes can also be dependent on the external stimulus, which was remarkably changed by temperature and/or Se supply (Krishnan et al., 2008; Ni et al., 2009; Bardil et al., 2011).

As a result of chilling stress, the plants are submitted to osmotic constrictions due to the reduced uptake of water. Then, the soil water potential progressively decreases, hampering and eventually halting the gradient of water flow from roots to apical shoot. The resulting osmotic stress may cause stomatal closure, reduced photosynthesis rate, growth inhibition, and ROS accumulation (Trovato et al., 2008). A response to osmotic stress widespread in plants consists in the accumulation of compatible osmolytes, such as Pro, which are thought to protect cells against stress damage.

The catabolism of Pro occurs in the mitochondria and it is connected to oxidative respiration and administers energy to resume growth after stress. During energy depletion, Pro might be oxidated to glutamate by flavin-dependent proline-dehydrogenase (PRODH) and NAD^+-dependent P5C dehydrogenase (P5CDH), which are two enzymes found in the mitochondria (Liang et al., 2013; Qamar et al., 2015; Zhang and Becker, 2015). Thus, the oxidation of Pro contributes to mitochondrial metabolism and ATP production by providing carbon skeletons and saving extreme energy depletion (Hildebrandt et al., 2015). The Pro behavior in this trial is supported by its negative correlation with TSS, and Suc during the stress with *C. canephora*, which showed $R^2 = -0.77$ and $R^2 = -0.79$, respectively ($p < 0.05$) (**Supplementary Figure 4**). In this case, the *C. canephora* reduced the Pro content during the stress to maintain the carbohydrates contents as an energetic source, avoiding carbohydrate starvation.

The PCA showed that Se supply responses vary not only in the species but also in different temperature conditions. It is also important to highlight that none of the analyzed variables showed a positive correlation with Se content in leaves during stress with *C. canephora* plants according to PCA1 (28.4%) and PCA2 (32.5%) (**Figure 4D**). **Figure 4D** shows that the variables analyzed presented a neutral or negative correlation with Se content. The absence of positive correlation during the stress is probably due to metabolic dysfunctions in *C. canephora* during low temperature, which resulted in higher injuries in the leaves.

Plant cells can sense chilling stress through low-temperature-induced changes in membrane fluidity, protein, nucleic acid conformation, and/or metabolite concentration (a specific metabolite or redox status) (Chinnusamy et al., 2007). Low temperature can inhibit the activities of some antioxidant enzymes (e.g., GR) that protect plants against ROS. The reduction of GR during the low temperature was not observed in the treatment of *C. canephora* without Se application. In this treatment, the GR increased 78% during the chilling stress compared with the same treatment before the stress (**Supplementary Table 1**). However, after the chilling stress, the Se application promoted three times more GR activity in plants when compared with those that did not receive Se. In other words, plants without Se were unable to maintain the GR activity after chilling stress.

Conclusion

Our findings showed a considerable depletion of plant metabolism at low temperature in both of the species studied, resulting in leaf damage and lipidic peroxidation (MDA), notably higher in *C. canephora*. The cold makes plants unable to trigger metabolic responses during the stress, reducing the content of carbohydrates and AA. Despite this, foliar Se application improved plants' odds of survival and reduced the leaf's injuries largely through enhancement in increasing the content of carbohydrates (TSS, starch, and Suc) and AA in the rewarming. All these compounds might also work as cryoprotective substances toward cold-sensitive enzymes, avoiding high membrane rigidity and also maintaining the membrane structure. Therefore, the application of Se at lower levels could be suggested as an important strategy for improving coffee development during cold, helping the plants to recover from the low-temperature stress. New trials focused on the impact of Se on gene expression and associated thermotolerance should be conducted to elucidate the role of this beneficial element on plant metabolism aiming at clarifying these results.

Data availability statement

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

Author contributions

GS, MS, RO, AC-J, and LG designed the research. GS, MS, GZV, and GAV conducted the experiments and chemical analyses. GS, MS, and EM analyzed the data. GS and MS wrote the original draft. RO, PB, DA, AC-J, and LG wrote the final text and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1000430/full#supplementary-material

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Soil and foliar selenium application: Impact on accumulation, speciation, and bioaccessibility of selenium in wheat (*Triticum aestivum* L.)

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A comprehensive study in selenium (Se) biofortification of staple food is vital for the prevention of Se-deficiency-related diseases in human beings. Thus, the roles of exogenous Se species, application methods and rates, and wheat growth stages were investigated on Se accumulation in different parts of wheat plant, and on Se speciation and bioaccessibility in whole wheat and white all-purpose flours. Soil Se application at 2 mg kg^{-1} increased grains yield by 6% compared to control (no Se), while no significant effects on yield were observed with foliar Se treatments. Foliar and soil Se application of either selenate or selenite significantly increased the Se content in different parts of wheat, while selenate had higher bioavailability than selenite in the soil. Regardless of Se application methods, the Se content of the first node was always higher than the first internode. Selenomethionine (SeMet; 87–96%) and selenocystine ($SeCys_2$; 4–13%) were the main Se species identified in grains of wheat. The percentage of SeMet increased by 6% in soil with applied selenite and selenate treatments at 0.5 mg kg^{-1} and decreased by 12% compared with soil applied selenite and selenate at 2 mg kg^{-1}, respectively. In addition, flour processing resulted in losses of Se; the losses were 12–68% in white all-purpose flour compared with whole wheat flour. The Se bioaccessibility in whole wheat and white all-purpose flours for all Se treatments ranged from 6 to 38%. In summary, foliar application of 5 mg L^{-1} Se(IV) produced

wheat grains that when grounds into whole wheat flour, was the most efficient strategy in producing Se-biofortified wheat. This study provides an important reference for the future development of high-quality and efficient Se-enriched wheat and wheat flour processing.

KEYWORDS

bioaccessibility, wheat, flour yield, selenium speciation, selenate, selenite

Highlights

- Foliar and soil Se application accumulated higher Se in different parts of wheat.
- first node plays an important role in transferring Se from xylem to phloem.
- SeMet (87–96%) and $SeCys_2$ (4–13%) were the main Se species in wheat grains.
- White all-purpose flours caused 12–68% Se lost compared with whole wheat flour.
- Bioaccessibility of different flour extraction rate (70 and 100%) was 6–38%.

Introduction

Selenium (Se) is an indispensable component of more than 25 Se-containing proteins involved in vital metabolic processes, such as metabolic enzyme thioredoxin reductase (TrxR), iodothyronine deiodinases (DIO), and antioxidant glutathione peroxidase (GSH-Px; Dong et al., 2020). It was estimated that at least one billion people have insufficient Se intake in the world (Zhang et al., 2019), which may cause various diseases in humans, including hypothyroidism, susceptibility to infection, tumors, rheumatoid arthritis, or heart failure (Rayman, 2012). The recommended daily dietary intake of Se generally ranges 50–55 $\mu g\ day^{-1}$ (WHO, 2004). Considering that organisms can't synthesize Se autonomously, human Se intake is primarily from the dietary diet, and food chains strategies to improve Se content in food crops can be achieved through Se biofortification practices (D'Amato et al., 2020; Wang et al., 2021; Zhou F. et al., 2021). Up to now, research on Se biofortification has been conducted in different plants including potato (Zhang et al., 2019; Dong et al., 2021), mushroom (Dong et al., 2020; Zhou et al., 2020), maize (Muleya et al., 2021), wheat (Liang et al., 2020; Xia et al., 2020; Wang et al., 2021), and rice (D'Amato et al., 2018).

Currently, soil and foliar Se application are widely used due to their simplicity and practicability (Dinh et al., 2019; Wang et al., 2020). In general, for soil Se application, there are interactions between the soil and Se, before it is absorbed by plant roots, and transported through xylem to storage parts, leaves, and subsequently to grains, i.e., wheat, via phloem (Li et al., 2008; Ducsay et al., 2016; Gupta and Gupta, 2017; Wang et al., 2019). Selenium can also enter the leaves after foliar Se application by penetrating through the cuticle or via the stomatal pathway (Saha et al., 2017). It is then transported to the edible parts of plant but its re-translocation relies on the nutritional status and phenological stage of plant (Saha et al., 2017; Connor et al., 2018). In cereal crops like wheat, the maturity of leaves determines whether a leaf competes with grain as a sink of Se or whether it can act as a source for Se translocation to grains. Mature leaves can only transport Se directly via phloem to grains but can't import Se (Saha et al., 2017). Thus, both soil and foliar Se application methods may enhance the transport of Se to the edible parts of plants (Boldrin et al., 2018). Recent studies suggested that foliar Se application at later growth stages is more effective for increasing the Se content of plant (Deng et al., 2017; Dinh et al., 2019; Wang et al., 2020). Nevertheless, the systematic study on the accumulation of Se in wheat grains with different Se application methods is still lacking.

No specific Se uptake pathways in plants are found yet since Se is not an essential element for plants (Dinh et al., 2019). Meanwhile, due to similar chemical properties between Se and sulfur (S), the uptake of Se(VI) occurs along the same pathway as sulphate, which occurs mainly through *SULTR1;1* and *SULTR1;2* transporters using an active transport process (Izydorczyk et al., 2020). Se(IV) is taken up by roots as $HSeO_3^-$ by the members of phosphate transporter *Pht1* family using aquaporins (Zhang et al., 2014; White, 2018).

Wheat is one of the staple crops for more than one third of the world's population (Boldrin et al., 2018), and is the most efficient Se accumulator among the common cereals (Poblaciones et al., 2014; Dinh et al., 2018). Nevertheless, 63% of the wheat grown in China, for example, is deficient in Se with an average concentration at 64.6 μg Se kg^{-1}, which provides insufficient daily Se for sustaining human health (Liang et al., 2020). Thus, agronomic Se biofortification of wheat may be one of the best approaches to increase Se intake by human. Studies showed that most Se absorbed by wheat was distributed in the grains (Keskinen et al., 2010; Eiche et al., 2015; Wang et al., 2017). Past research efforts generally focused on Se content in

root, grain, leaves, stem, and glume of wheat (Lyons et al., 2005; Shinmachi et al., 2010; Eiche et al., 2015; Nawaz et al., 2015; Liu et al., 2016; Wang et al., 2020). Importantly, the rachis and nodes may also play important roles in transporting Se from leaves and roots to the developing grains in panicles and transferring Se from the xylem to phloem, respectively (Chen et al., 2018). However, there is no systematic study on the effects of different Se application methods on Se accumulation in various parts of wheat, especially in nodes and rachis.

Selenomethionine (SeMet) is the primary Se species in wheat grains (Poblaciones et al., 2014; Eiche et al., 2015; Wang et al., 2020). Lu et al. (2018) also reported that in Se-enriched wheat, SeMet accounted for 44.2% of the total Se, while selenocystine ($SeCys_2$) and methylselenocysteine (MeSeCys) accounted for 2.6 and 0.3% of the total Se, respectively (Carey et al., 2012). In general, most exogenous Se was accumulated in wheat leaves after foliar Se application (Wang et al., 2020), however, some research questions remain. For example, is there a correlation between Se speciation in leaves and grain of wheat? Is the Se speciation in these tissues affected by different Se application methods, rates, species, and growth stages of wheat?

The production of Se-enriched wheat can be an important step in eliminating the negative impact of Se deficiencies in low Se areas. In this regard, bioaccessibility of Se from edible wheat tissues is important to understand. The bioaccessibility of Se using in vitro simulated gastrointestinal digestion test (PBET) refers to the portion of a nutrient, e.g., Se in a food product, that can be found dissolved in gastric (G) and intestinal (I) phases, and be potentially absorbed and utilized by organisms (Zhou F. et al., 2021). The order of bioaccessibility of Se for different gastrointestinal digestion simulation methods in gastricand intestinal was as follows: PBET > UBM (unified bioaccessibility method) > SBRC (solubility bioaccessibility research consortium method) > IVG (in vitro gastrointestinal method; Zhou et al., 2020). The PBET method has become a common evaluation method for evaluating the bioaccessibility of Se (Zhou et al., 2019; Muleya et al., 2021). Hitherto many studies on Se bioaccessibility in green vegetables have been carried out, including on Se-enriched leeks (Lavu et al., 2012), potato (Dong et al., 2021), lettuce (Do et al., 2017), and Se-enriched crops, such as maize (Muleya et al., 2021). For example, Muleya et al. (2021) found that the mean bioaccessibility of Se was 73.9 ± 8.5% with no significant difference across all selected crops (maize, groundnut, and cowpea). Especially, Lu et al. (2018) showed that the bioaccessibility of Se in Se-enriched wheat and soybeans was 90%, corn and broccoli was 80%, and cardamine was 50%. In wheat, however, the embryo and endosperm are the main storage sites of Se in wheat grain, about 80–90% of Se is stored in wheat flour after grinding the grains (Lyons et al., 2005), and nearly 5% of the whole grain Se was lost in the milling process (Govasmark et al., 2010). To date, it has not yet been reported whether the Se bioaccessibility in whole wheat and in white all-purpose flours is significant different, and whether Se application methods affect the Se bioaccessibility.

Currently, the main methods used to explore the uptake, translocation, and transformation of Se in crops can be divided as: hydroponic experiment, pot experiment, and field experiment (Wang et al., 2019, 2020, 2021; Xia et al., 2020). The environment for hydroponic experiments is quite different from the actual soil environment, which is completely different from field experiments (Wang et al., 2019). It is difficult to analyze the environmental process and influencing factors of field experiments, since the conditions of field experiments are not well controlled, and temperature and humidity will affect the experimental results (Wang et al., 2020; Xia et al., 2020). Pot experiment can both study the mechanism and be closer to the actual soil environment (Wang et al., 2021). Given above, the pot experiment is more suitable at present and can accurately explore the reality. Wheat, as a world-wide consumed crop that has a strong Se accumulation ability (Wang et al., 2021), was selected as the research crop in this study. We hypothesized that different Se application treatments will affect the growth of wheat and then influence the Se speciation and Se bioaccessibility in Se-enriched wheat flours. The main objectives of this study were as follows: (1) compare the effects of different Se application methods on the growth and Se accumulation in different parts of wheat; (2) explore the influences of two Se application methods on the Se speciation in the leaves and grains of wheat; and (3) ascertain the differences of Se bioaccessibility under different Se treatments in whole wheat and white all-purpose flours.

Materials and methods

Materials and reagents

The pot experiment was carried out in a greenhouse at Northwest Agriculture and Forestry University in Yangling, Shaanxi from year 2018 to 2019. Tested soil was collected from the non-polluted farmland around Northwest A&F University, which has never received applied exogenous Se. After air-drying, homogenizing, and grinding, the soil was passed through 2 and 0.149 mm sieve for physical and chemical analysis determined according to Bao (2000). The relevant physicochemical properties are as follows: soil pH, 8.14; carbonate content, 118.0 g kg^{-1}; organic carbon, 8.53 g kg^{-1}; cation exchange capacity, 23.34 cmol(+) kg^{-1}; amorphous aluminum, 0.40 g kg^{-1}; amorphous iron, 1.20 g kg^{-1}; clay, 39.6%; and total Se, 0.139 mg kg^{-1}.

Winter wheat seeds (*Triticum aestivum* L, Xiaoyan-22) were provided by a commercial seed company of Northwest Agriculture and Forestry University. Wheat seeds with full

grains were selected for consistent size and no pest infestation damage, then disinfected with 5% (V/V) H_2O_2 for 30 min and washed thrice with deionized water.

Se(IV) was sodium selenite (Na_2SeO_3, ≥ 97%; Tianjin Fuchen Chemical Reagent Factory), and Se(VI) was sodium selenate (Na_2SeO_4, ≥ 98%; Beijing Xiya Chemical Industry Co., Ltd), both were analytical pure reagents. The organic Se (SeMet, $SeCys_2$, and MeSeCys) were all purchased from Sigma-Aldrich company and used for the determination of Se speciation in grain and leaves of wheat. Pepsin, sodium malate, sodium citrate, lactic acid, acetic acid, bile salt and trypsin, which were used for the determination of Se bioaccessibility, were all purchased from Yuanye Biological Technology Co., Ltd.

Experimental design

A complete block design was used in this study, two species of exogenous Se (Se(IV) and Se(VI)), three application rates of Se (0.5, 1, and 2 mg kg^{-1}) were selected in soil Se application. For foliar Se treatments, two species of exogenous Se (Se(IV) and Se(VI)), three application rates of Se (5, 10, and 20 mg L^{-1}) were applied at two growth stages of pre-flowering stage (F1) and pre-filling stage (F2). A total of 19 treatments were used in this experiment and each treatment was replicated three times.

Pots had an inner diameter of 32 cm and a depth of 20 cm, and were filled with 8 kg soil. All soil samples were completely air-dried, ground, and prepared through a 5 mm sieve. Se(VI) and Se(IV) solutions were prepared according to the designed Se application rates and then evenly sprayed into the soil and mixed. The soil moisture was adjusted to 70% of the water holding capacity. After full mixing, the sprayed soil was allowed to equilibrate at 25°C for 30 days (Li et al., 2016), and deionized water was added every 2–3 days during the equilibrium stage.

During the sowing period, 0.15 g N (urea, analytical pure) and 0.033 g P (potassium dihydrogen phosphate, analytical pure) were applied to each kilogram of soil, and 0.15 g kg^{-1} nitrogen fertilizer was applied at regreening stage of wheat. 20 seeds were sowed into each pot. Two weeks after the emergence of seedlings, the seedlings were thinned to 10 plants per pot. The pots were weighed and watered every 4–14 days during the wheat growing season. For the foliar Se application, the Se solution was sprayed evenly on the plants during the pre-flowering stage (April 2019) and the pre-filling stage (May 2019) of growth. Specifically, 100 mL Se (IV) or Se (VI) solution (5, 10, and 20 mg L^{-1}) were mixed into water with 0.1% surfactant. Foliar Se was applied three times (100 mL each time, respectively) in intervals of 5 days to ensure that Se was fully absorbed by wheat leaves. Each pot was sprayed with a total of 1.5, 3, and 6 mg Se(IV) and Se(VI), respectively. Moreover, during the foliar application process, the soil surface was covered with plastic film to avoid Se from dripping onto the soil.

Sample collection

The height and length of rachis and the effective ear number of wheat were measured after wheat harvest (June 2019). The harvested wheat was first washed with tap water thrice to remove dust and other impurities, rinsed with deionized water thrice, and then dried with absorbent paper. Meanwhile, each wheat plant was divided into nine parts: root, stem, leaf, glume, grain, sheath, first internode, first node, and rachis (Chen et al., 2018). After weighing fresh weight (FW) of roots, each replicate was placed into paper bags, dried at 90°C for 30 min and at 55°C for 3 days, and then dry weight (DW) was recorded. All parts of wheat were ground into powder to determine the total Se content. In addition, fresh grain and leaf tissue samples were freeze-dried, grounded, and then stored at 4°C for the determination of Se speciation (described later). Flour and bran were separated by a sieve (0.149 mm), weighed, mixed to obtain whole wheat and white all-purpose flours, and ground into powder for the determination of Se bioaccessibility (see section "*In vitro* simulated gastrointestinal digestion test").

Determination of samples

Selenium content in various parts of wheat

The total Se content was determined via hydride atomic fluorescence spectrometry (AFS, Beijing Jitian AFS-930 dual-channel atom fluorescence photometer, Beijing, China) after wet-acid digestion. The specific procedure has been described by Wang et al. (2020).

Selenium speciation in wheat grains and leaves

Selenium speciation was determined by HPLC-ICP-MS. First, 0.2000 g freeze-dried grains or leaves was taken into a centrifuge tube, 20 mg protease XIV and 5 mL water were added, vortexed for 30 s, ultrasonic extraction for 3 h in a 37°C water bath and shook several times during the period. Second, the sample was centrifuged at 9,000 r min^{-1} for 10 min at 4°C. The supernatant was collected after pouring through a 0.22 μm filter membrane, and then analyzed using the HPLC-ICP-MS system. The instrument conditions are as follows: for the HPLC; Hamilton PRP-X100 anion exchange column (250 mm × 4.1 mm, 10 μm) was used, the column temperature was room temperature, the mobile phase was 40 mmol L^{-1} diammonium hydrogen phosphate (pH = 6.0 adjusted with 10% formic acid) at a flow rate of 1.0 mL min^{-1}, and the injection volume was 100 μL. For the ICP-MS; RF power was 1,550 W, RF matching voltage was 1.8 V, sampling depth was 8 mm, atomization chamber temperature was 2°C, plasma gas flow rate was 15.0 L min^{-1}, the flow rate of carrier gas was 0.65 L min^{-1}, the mode was high He collision mode, the flow rate of collision gas was 4.5 mL min^{-1}, peristaltic pump speed was 0.3 r s^{-1}. The detection mass number m/z = ^{78}(Se), and the integration

time was 0.5 s. At the same time, Se-enriched yeast of SELM-1 was used as the quality control sample, the measured content of SeMet in the quality control sample was 3,236 ± 21 mg kg^{-1}, the standard value was 3,389 ± 173 mg kg^{-1}, the recovery rate was 95.5%.

In vitro simulated gastrointestinal digestion test

According to the method of Zhou et al. (2019), the PBET method was carried out, and divided into two stages: gastric (G) digestion and intestinal (I) digestion. The specific steps are as follows:

(1) G: 1.000 g sample was accurately weighed into 100 mL polyethylene centrifuge tube, and 50 ml fresh gastric juice (pH 2.5) were added into a constant temperature (37°C) water bath for digestion at 150 rpm for 1 h. The obtained digestive juice was centrifuged at 4,000 rpm for 10 min and 10% of the supernatant was removed and stored at 4°C for Se content determination.
(2) I: the pH of the remaining digestive juice was adjusted to 7.0 by 10% (m/v) NaOH. Then 5 mL intestinal fluid were added and digested in a constant temperature water bath (150 rpm, 37°C) for 4 h. The obtained digestive fluid was centrifugated at 4,000 rpm for 10 min, and the supernatant was stored at 4°C for further Se content determination.

Moreover, the total Se content in G and I sample (2 mL of digestive fluid) were determined by the method already described in section "Selenium content in various parts of wheat." The composition of gastric juice and intestinal juice was the same as Zhou et al. (2019).

Statistical analysis

Pearson correlation analysis and variance analysis were performed by SPSS 20.0 (IBM, United States; Duncan method was used for significance test at $\alpha = 0.05$). The data in the chart are the averages of three replicates, and the data were calculated using the following Eqs.

$$TF_{a-b} = \frac{C_b}{C_a} \quad (1)$$

where TF_{a-b} represents the Se translocation factor from part "b" to part "a" of wheat (Dinh et al., 2019). C_a and C_b represent the Se content in part "a" or part "b" of wheat, respectively (μg g^{-1}). "a" or part "b" refer to different parts of "root," "first node," "rachis," "grain," and "leaves."

$$BA\% = \frac{\text{Se in G/I}}{\text{Se in sample}} \times 100\% \quad (2)$$

where BA% represents the Se bioaccessibility of whole wheat and white all-purpose flours. Selenium in G/I was the Se content in gastric or intestinal phase of the sample, mg kg^{-1}. Se in sample indicates the Se content in the corresponding sample, mg kg^{-1}.

$$LS\% = \frac{\text{lost Se content}}{\text{Se in sample}} \times 100\% \quad (3)$$

where LS% represents the Se lost proportion with different flour yield. The lost Se content (μg g^{-1}) is the difference between the Se content of whole wheat and white all-purpose flours with different Se treatments.

Results

Basic growth index of wheat

Figure 1 shows the growth of wheat at different growing stages. Albino seedlings appeared at tillering stage when 2 mg kg^{-1} Se(VI) was soil applied, indicating that the 2 mg kg^{-1} Se treatment has little inhibition on wheat growth. However, the growth of wheat appeared to slow down due to the biological dilution effect at the later growth stage of wheat.

Supplementary Table 1 illustrated that different Se treatments had significant ($p < 0.05$) effects on the biomass and grain yield of wheat. Soil application with 2 mg kg^{-1} Se(IV) resulted in the highest grain yield of wheat, which was about 6% higher than control treatment. Compared with control, all soil Se application treatments reduced the yield of wheat (by 4–5% by Se(IV) treatments, except at 2 mg kg^{-1} Se(IV) (yield increased by 10% compared with 0.5 mg kg^{-1} treatments), and 4–62% in Se(VI) treatments). However, no significant effects ($p > 0.05$) were observed in the grain yield of wheat at different foliar Se application treatments, irrespective of the Se species, application rates, and application stages.

We note that soil Se(IV) application treatments significantly ($p < 0.05$) increased the biomass of wheat (7–11%), compared with control, while the application of Se(VI) increased Se application rates both significantly ($p < 0.05$) reduced wheat biomass (2–59%). Compared with 2 mg kg^{-1} Se(VI) treatment, the biomass of wheat treated with 0.5 mg kg^{-1} Se(VI) increased by 58%. No significant ($p > 0.05$) effects on the biomass of wheat were found among different foliar Se application treatments, irrespective of exogenous Se species, application rates, and application stages.

Selenium content in wheat grain

The harvested wheat plants were divided into nine parts: root, stem, leave, sheath, first internode, first node, rachis, grain, and glume (**Figure 2**). We observed that application of Se, either via foliar or soil methods, significantly ($p < 0.05$) increased the Se content in each part of wheat in comparison to control. The Se content increased with higher rate of Se application.

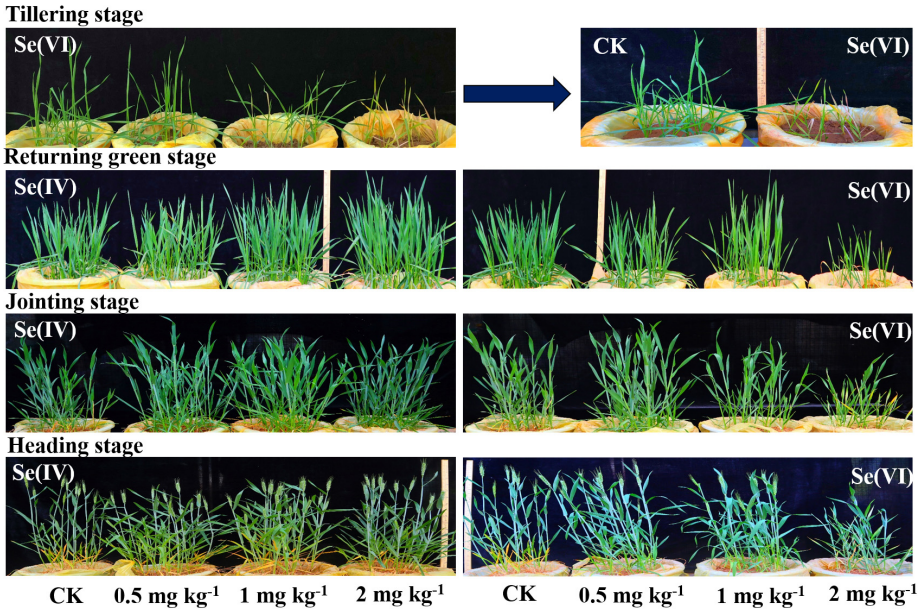

FIGURE 1
The growth of wheat at different growth stages under different Se treatments. Se(IV) refers to selenite treatment and Se(VI) refers to selenate treatment.

A significant ($p < 0.05$) increase of 72 and 84% of the Se content in grains of wheat was observed at soil application 2 mg kg^{-1} Se(IV) and Se(VI) treatments, compared with 0.5 mg kg^{-1} treatment of either form. Meanwhile, the Se content in grains with foliar application rate of 20 mg L^{-1} Se(IV) and Se(VI) increased remarkably ($p < 0.05$) by 68–69% and 60–68% at pre-flowering and at pre-filling stages, respectively, compared with the corresponding 5 mg L^{-1} Se application treatments.

Regardless of application method of Se(IV) or Se(VI), the Se content of the first node of wheat was always higher than that of the first internode. Meanwhile, irrespective of the Se application rate and method, Se(VI) treatments significantly ($p < 0.05$) increased the Se content in each part of the wheat (90–99.5%), compared with Se(IV) treatments (except foliar application of 20 mg L^{-1} Se(VI) applied at pre-filling stage). Compared with foliar Se(IV) treatment, foliar application of Se(VI) at pre-flowering stage and pre-filling stage significantly ($p < 0.05$) increased the Se content of wheat grains by 6–44% and 3–28%, respectively. In addition, the Se content of wheat grains from foliar Se(IV) and Se(VI) application at pre-filling stage significantly ($p < 0.05$) increased by 22–30% and 6–25% than that applied at pre-flowering stage, respectively.

Foliar Se(IV) application significantly ($p < 0.05$) increased the Se content of wheat grains compared with corresponding soil application treatments, irrespective of the application stages. Specifically, the Se content of wheat grains sprayed with Se(IV) significantly ($p < 0.05$) increased by 45–54% and 61–68% at pre-flowering and pre-filling stages, respectively, compared with soil Se(IV) application. In contrast to foliar Se(IV) treatment, soil application of Se(VI) significantly ($p < 0.05$) increased the Se content of wheat grains compared with its foliar application. The Se content of wheat grains in the soil application treatments was significantly ($p < 0.05$) increased by 79–91% and 75–90% at the pre-flowering and pre-filling stages, compared with foliar application treatments, respectively.

Translocation factor of Se in wheat plant

Translocation factor (TF) can be used to reflect the translocation capacity of plant from source to sink (Dinh et al., 2019). **Figure 3** showed the effects of different Se treatments on TF among different parts of wheat. According to the different Se application methods (soil and foliar Se application), the TF of Se in wheat was divided into two parts: (a) soil Se application: $TF_{first\ nodes/root}$, $TF_{rachis/first\ nodes}$, and $TF_{grains/rachis}$, (b) foliar Se application: $TF_{root/first\ nodes}$, $TF_{rachis/first\ nodes}$, $TF_{grains/rachis}$, and $TF_{grains/leaves}$.

Compared with control, soil Se application treatments significantly increased the $TF_{grain/rachis}$ of wheat (1.2–2.1), while reduced the $TF_{rachis/first\ nodes}$ (0.6–1.1) and $TF_{first\ nodes/root}$ (0.4–0.7; except at the soil application of 2 mg kg^{-1} Se(VI)). Moreover, when Se(IV) was soil applied, the $TF_{grains/rachis}$ increased with the higher application rate of exogenous Se, and the $TF_{grains/rachis}$ decreased when Se(VI) was soil applied. Although soil Se application reduced the $TF_{first\ nodes/root}$, $TF_{first\ nodes/root}$ increased with a higher rate of exogenous soil

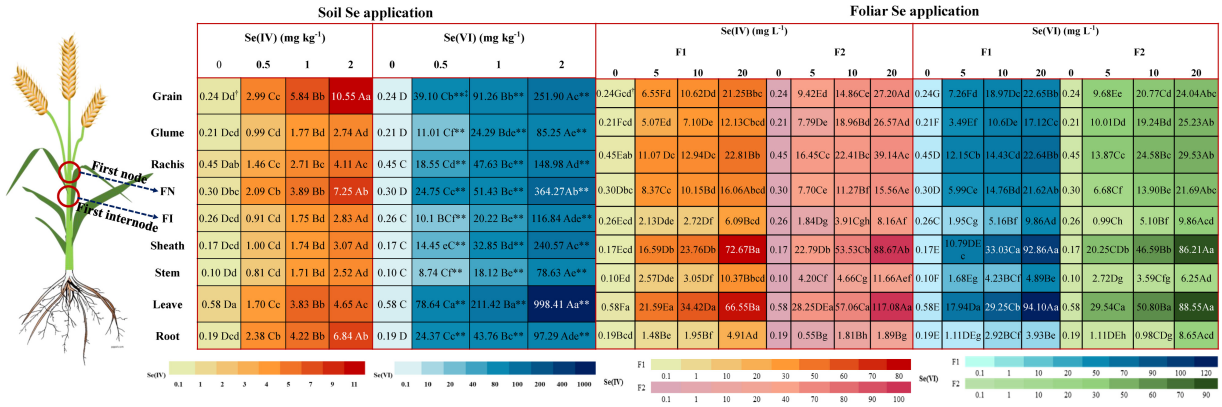

FIGURE 2

The Se content in different parts of wheat under different soil and foliar Se treatments. F1 represents pre-flowering stage and F2 represents pre-filling stage. Different lowercase letters of "a"–"h" indicate significant ($p < 0.05$) differences between different parts of wheat at each treatment. Different capital letters of "A"–"G" indicate significant ($p < 0.05$) differences between different rates of soil Se(IV), soil Se(VI), foliar Se(IV), and foliar Se(VI) application on Se concentration in the same parts of wheat ($p < 0.05$). "**" and "*" indicate the significant ($p < 0.01$; $p < 0.05$) differences between the same part of wheat and the same Se application rates of different Se treatments, respectively.

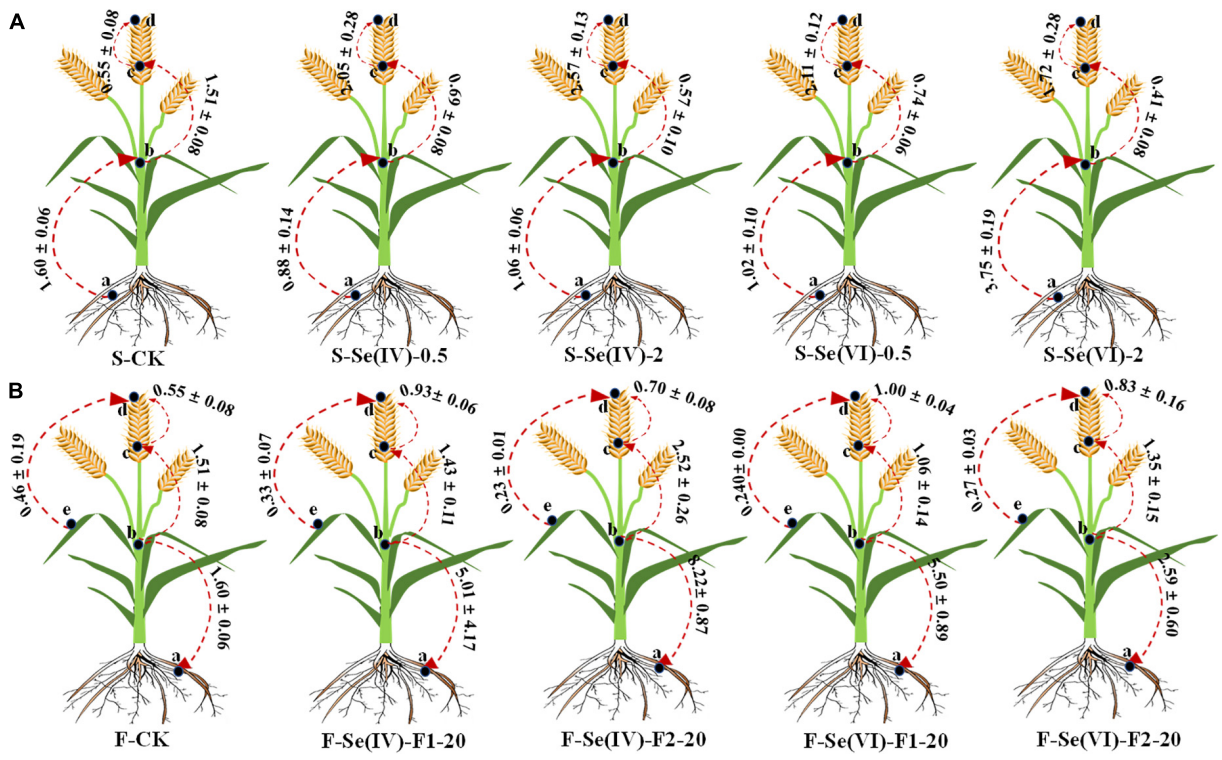

FIGURE 3

The TF values of wheat under different soil and foliar Se treatments. CK = control, F1 represents pre-flowering stage and F2 represents pre-filling stage; **(A)** represents TF of wheat with soil Se application, **(B)** represents TF of wheat with foliar Se application, "a," "b," "c," "d," and "e" denote different parts of "root," "first node," "rachis," "grain," and "leaves," respectively. The direction of the arrow indicates the direction of TF.

Se applied, irrespective of the Se species. Regardless of the application stages, rates, and species of Se, foliar spraying of exogenous Se had no significant ($p > 0.05$) effects on $TF_{grains/leaves}$. Compared with control, foliar Se application of both forms of Se increased the $TF_{grains/rachis}$ (0.1–0.8) in wheat. Compared with pre-filling stage, the $TF_{grains/rachis}$ in wheat increased at pre-flowering stage ((Se(IV): 0.2–0.5; Se(VI): 0.1–0.9)). In addition, spraying Se(IV) at pre-filling stage

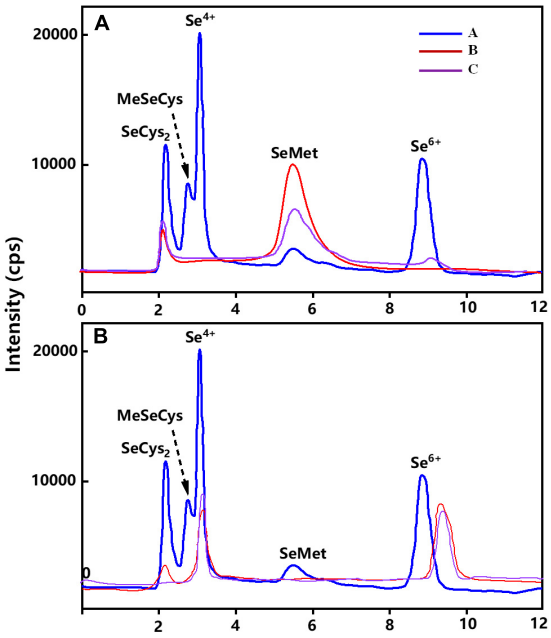

FIGURE 4
Chromatogram of Se species in wheat grain and leaves under different soil and foliar Se treatments. **(A)** represents the Se species in wheat grain and **(B)** represents the Se species in wheat leaves. "A" represents Se species in wheat grain in standard compounds, "B" represents Se species in wheat grain under soil application of Se(IV), and "C" represents Se species in wheat grain under soil application of Se(VI).

significantly ($p < 0.05$) increased the $TF_{rachis/first\ nodes}$ (0.8–1.1) and the $TF_{root/first\ nodes}$ (0.9–8.4), compared with control.

Selenium speciation in grains and leaves of wheat

Chromatogram of wheat grains and leaves

Figure 4A showed the percentages of Se species or Se compounds identified in wheat grains under different Se treatments. Irrespective of the Se application methods, Se speciation in wheat grains treated with different forms of exogenous Se were mainly organic Se (93–100%). Organic Se was mainly composed of SeMet (87–96%) and $SeCys_2$ (4–13%) (Figure 5), while Se(VI) was the main inorganic Se species in wheat grains (1–6%). Figure 4B represents the chromatogram of wheat leaves, it can be seen that Se(VI) was the main Se species in wheat leaves.

Distribution of Se speciation in grains of wheat

No significant differences were observed for the percentages of SeMet in wheat grains among soil (83–95%) and foliar (87–96%) Se application treatments, while the percentages of SeMet varied with Se application rate (Figure 5). The percentage of SeMet in soil Se(IV) and Se(VI) treatments at 0.5 mg kg^{-1} increased by 6% and decreased by 12%, respectively, compared with the 2 mg kg^{-1} treatment, respectively. However, there was no significant ($p > 0.05$) differences among the foliar Se application rates.

The species of exogenous Se also affected the percentages of SeMet in grains. With soil application rate at 0.5 mg kg^{-1}, the percentage of SeMet in wheat grains increased by 7% in Se(VI) treatment compared with Se(IV) treatment, but decreased by 11% at 2 mg kg^{-1} Se(VI) soil treatment. However, the percentage of $SeCys_2$ in wheat grains increased by 9% with 2 mg kg^{-1} soil Se(VI) treatment compared with 0.5 mg kg^{-1} Se(VI) soil treatment. Moreover, foliar Se(IV) application increased the percentage of SeMet in wheat grains, compared with Se(VI) application. We found that compared with Se(VI) treatments, the percentage of SeMet in wheat grains treated with Se(IV) increased by 3–7%. The percentage of SeMet measured in wheat grains produced from foliar Se treatment with high rate of Se(VI) at pre-flowering and pre-filling stages, increased by 9 and 5%, respectively, compared with soil Se treatments.

Distribution of Se speciation in leaves of wheat

Regardless of the application methods, inorganic Se (50–100%) was the major Se species in wheat leaves in all the treatments, with Se(IV) and Se(VI) accounting for 1–71% and 18–99% of total Se, respectively, while SeMet (1–42%) was the main organic Se species (Figure 4B). Moreover, Se(IV; 20–71%) and Se(VI; 85–99%) were the main Se species in wheat leaves when treated with Se(IV) or Se(VI) application via foliar or soil Se application, respectively. In addition, the percentage of Se(IV) was reduced by 31–39% after foliar Se(IV) application at pre-flowering stage, but the percentage of Se(VI) was increased by 34–39%, compared with pre-filling stage. Compared with Se(IV) treatments, the perecentage of SeMet increased by 12% and 14% in wheat leaves applied with Se(VI) at pre-flowering stage and pre-filling stages, respectively. The percentages of SeMet were increased at pre-filling stage compared with pre-flowering stage. Meanwhile, a 10% and 3% increase was observed in leaves at 20 mg L^{-1} Se(VI) treatment at pre-flowering stage and pre-filling stage, respectively, compared with foliar Se(IV) application.

Irrespective of the application methods of Se, the percentage of organic Se in wheat leaves with soil application at 2 mg kg^{-1} Se(IV) rate was the highest, which was 35–49% higher than other treatments. Except for soil Se(IV) application at 2 mg kg^{-1}, the percentage of organic Se in wheat leaves sprayed with 20 mg L^{-1} Se(VI) increased by 8% and 11% at pre-flowering and pre-filling stages, respectively. The percentage of SeMet in wheat leaves with soil Se(IV) application at 2 mg kg^{-1} increased by 40% compared with the corresponding foliar application treatments at pre-flowering stage. The percentage of Se(VI) in wheat leaves

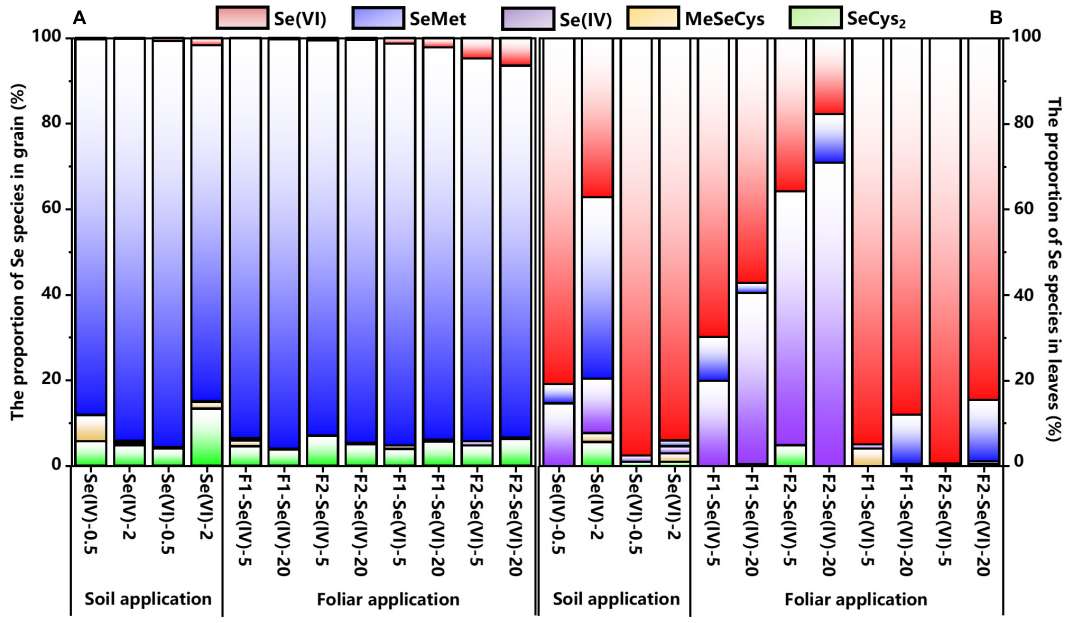

FIGURE 5
The proportion of Se species in wheat grain and leaves under different soil and foliar Se treatments. **(A)** represents the Se proportion in wheat grain and **(B)** represents the Se proportion in wheat leaves.

with soil application at 20 mg L^{-1} Se(VI) increased by 8% and 9% applied at pre-flowering stage and pre-filling stages, respectively, compared with foliar application treatments.

Selenium bioaccessibility in wheat flour

Selenium lost in whole wheat and white all-purpose flour

The whole wheat (100% wheat) and white all-purpose flour (70% wheat flour and 30% bran) were obtained by controlling different proportions of flour and bran of wheat. Selenium lost was calculated as the difference between the Se content of whole wheat and white all-purpose flour. Irrespective of the Se application methods, the percentage of Se lost in wheat flour process among the different Se treatments ranged from 12 to 68%. For soil Se application at 2 mg kg^{-1} Se(IV) and Se(VI) treatments, the percentages of Se lost in wheat flour were 4% and 8%, respectively, compared with the 0.5 mg kg^{-1} Se(IV) and Se(VI) treatments. The percentages of Se lost in wheat flour produced from plants sprayed with 5 mg L^{-1} Se(VI) increased by 40% (pre-flowering stage) and 23% (pre-filling stage), compared with 20 mg L^{-1} Se(VI) treatment.

In general, flour produced from foliar Se application had a higher percentage of Se lost in flour produced from soil Se application. The percentage of Se lost in wheat flour treated with foliar Se(IV) application was 2–12% (pre-flowering stage) and 43–51% (pre-filling stage) higher than that of the soil Se treatments (except for spraying Se(VI) at pre-filling stage). When Se(VI) was sprayed, the percentage of Se lost in wheat flour was 28% (at pre-filling stage) and 42% (at pre-filling stage) higher than the soil Se(VI) treatment.

The bioaccessibility of Se in wheat flour

The bioaccessibility of Se in whole wheat and white all-purpose flours are shown in Figure 5 for different Se treatments. The bioaccessibility of Se in white all-purpose flour was higher than that in whole wheat flour. In the gastric stage (G), the bioaccessibility of Se was 6–27% and 6–34% in whole wheat and white all-purpose flour, respectively. Meanwhile, the bioaccessibility of Se in whole wheat flour was 9–34% and 10–38% in white all-purpose flours in the intestinal phase (I).

Irrespective of the Se application methods and Se species, the Se bioaccessibility in wheat flour (either whole wheat and white all-purpose flours) produced from soil at 2 mg kg^{-1} increased by 6–13% compared with 0.5 mg kg^{-1} treatments, except for the soil application of 2 mg kg^{-1} Se(VI) treatments. Compared with the 0.5 mg kg^{-1} Se(VI) treatment, the Se bioaccessibility in whole wheat and white all-purpose flours decreased by 13% (G) and 16% (I), and 15% (G) and 17% (I) in soil at 2 mg kg^{-1} Se(VI) treatment, respectively. In addition, the Se bioaccessibility in Se(VI) treatments in both foliar and soil Se application was higher than that in Se (IV) treatment, except soil application at 2 mg kg^{-1} Se(VI). In soil Se application, the bioaccessibility of Se in wheat flour of Se(VI) treatment increased by 4% in

both G and I (in whole wheat flour), compared with Se(IV) treatment. Compared with foliar Se application at pre-flowering stage, foliar Se application at pre-filling stage increased the bioaccessibility of Se in whole wheat (3–4%) and white all-purpose flours (2–8%) in G and I (1–3%, whole wheat and 1–6%, white all-purpose flour).

Discussion

Effects of selenium application methods on the growth of wheat

Selenium has been reported to be a beneficial element that can promote plant growth and improve plant resistance to stress although the essentiality of Se to plants is still questionable (Schiavon et al., 2015). Others have reported that the excessive accumulation of Se in plants may also inhibit the growth of crops (Wang et al., 2019). In this study, we found that soil application at 2 mg kg^{-1} Se(VI) significantly reduced plant height, effective ear number, and rachis length of wheat compared with 0.5 mg kg^{-1} Se (VI) treatment (**Figure 1**, **Supplementary Figures 1**, **3**), indicating that 2 mg kg^{-1} Se(VI) has a certain toxic effect on wheat growth. Based on this observation, it appears that 1 mg kg^{-1} can be used as the tolerance limit of Se(VI) in a wheat Se biofortification strategy. The grain yield of wheat in 0.5 mg kg^{-1} Se(VI) treatments increased by 61% compared with 2 mg kg^{-1} Se(VI) treatments. Similarly, this study also found that the highest grain yield was significantly ($p < 0.05$) obtained in soil applied with 2 mg kg^{-1} of Se(IV), which was about 6% higher than the control treatment (**Supplementary Table 1**).

Previous studies have obtained varied results about different Se application methods. For example, Lara et al. (2019) and Ducsay et al. (2016) found that foliar Se application increased the yield of wheat. A two-year field study on the purple-grained wheat and common wheat showed that the soil Se application increased shoot dry weight and grain yield, while there was no significant ($p > 0.05$) difference between foliar Se application and control treatment. Zhang and Zhou (2019) found that neither foliar nor soil Se application had significant effects on rice yield and biomass ($p > 0.05$). However, in soil Se application, compared with the selenite application (1 mg kg^{-1}), the grain and the biomass yield of ZM-9023 significantly ($p < 0.05$) increased by about 15% for selenate application (10 mg kg^{-1}; Wang et al., 2021). The discrepancy in results may be attributed to the different growth stages and methods of Se application. Although soil Se application during the sowing period didn't affect the uptake efficiency of Se immediately (Curtin et al., 2006), Se can play a role in the entire growth cycle of wheat. Wheat can only absorb exogenous Se from pre-flowering stage or pre-filling stage to maturity stage in foliar Se treatments. Although foliar Se application in a wheat Se biofortification strategy is more efficient than soil Se application for increasing Se concentration in wheat, it has no significant effect on wheat yield.

The reason why application 0.5 mg kg^{-1} selenite increased yield may due to that soil Se application may influence the soil microorganisms and thereby promote the growth, development, and yield of wheat (the entire growth stage; Dinh et al., 2019). In addition, the increase of crop yield by exogenous Se application may be related to the improvement of crop's antioxidant capacity (D'Amato et al., 2018). Studies showed that applying appropriate rates of exogenous Se increased the antioxidant capacity of crops (Gupta and Gupta, 2017). The activities of SOD, POD, CAT, and other enzymes all increased with the application of exogenous Se is the main reason for the increased yields reported (Nawaz et al., 2015). However, high Se rate application can also be toxic to crops reduce their antioxidant capacity and yields (as we observed on decreased yield with high rate of Se (VI)). Therefore, application of appropriate rates of Se may reduce the oxidative stress and increase the biomass and yield of wheat. The underlying mechanisms of the increase in yield still need to be further studied.

Effects of selenium application on selenium uptake and translocation in wheat

Selenium content in wheat grains was higher with either soil or foliar Se application compared with control (**Figure 2**), which is consistent with the results of Keskinen et al. (2010) and Wang et al. (2019). They all found that most of the Se absorbed by wheat was distributed in the grain, indicating Se application can improve the Se content in grain. In this study, we separated wheat into nine parts (sheath, first internode, first node, and rachis haven't been systematically studied) for the first time. Consistent with previous studies (Nawaz et al., 2015; Boldrin et al., 2018), this study observed that soil application of Se(VI) significantly ($p < 0.05$) increased the Se content in each part of wheat (90–99.5%), and spraying Se(VI) increased the Se content of wheat grains (3–44%) compared with Se(IV) treatment. This increase in Se accumulation with selenate may be attributed to the different transport mechanism of Se(VI) and Se(IV) in plants. The uptake and translocation of these two inorganic forms of Se by plants is an energy-consuming process (Li et al., 2008). Due to the similar chemical properties between Se(VI) and sulfate, Se(VI) enters the roots of plants through the sulfate transport system (Shinmachi et al., 2010). Se(VI) absorbed by plants is easily transported from roots to shoots with no speciation change, it is reduced to Se(IV) in leaves, and then converted into organic Se compounds, which are then distributed to other plant tissues (Gupta and Gupta, 2017; Wang et al., 2020). However, Se(IV) is more easily converted into organic forms (including SeMet and its oxide, SeOMet) after

being absorbed by plant roots and mainly accumulate in root, only a small part can be transported to shoots (Li et al., 2008).

Rachis is the organ connecting the stem and grain of wheat (Chen et al., 2018). Selenium applied by fertilizers is absorbed by leaves (foliar applied) and roots (soil applied) of wheat and are eventually transported to the developing grains through the rachis of wheat. In this study, the Se content in the rachis and grains of wheat were higher than other parts of the plant for all treatments with foliar Se application (**Figure 2**). In this regard, recent studies suggested three pathways of foliar Se uptake, including cuticular, plant stomata, and trichomes (Zhou J. et al., 2021). Foliar application conditions can also affect the absorption of Se fertilizers (Shahid et al., 2017), and the leaf physical characteristics such as stomatal density, roughness, and epidermal wax layer, may affect the deposition of fertilizers on the surface of leaves (Chen et al., 2018). Compared with soil Se(IV) application, the grains of wheat treated with foliar application of Se(IV) have a higher Se content (**Figure 2**), indicating that foliar Se application can efficiently increase the translocation of Se to the grain, especially in the phloem (Adrees et al., 2015). The Se content of wheat is determined by the transport of xylem-mediated Se transport from the root to the aerial part and phloem-mediated Se (Kato et al., 2010).

This study found that irrespective of the Se application methods, the Se content in the first node was higher than that of the first internode. Except for wheat grains and leaves, the Se content in first internodes and nodes was relatively high, which was consistent with findings reported by Zhou J. et al. (2021). These results showed that the first node plays an important role in the storage of exogenous Se in wheat. Although no studies have explored the effect of application of exogenous Se on the gene expression in first nodes, it was speculated that the upregulation of transporter-related genes helped allocate the transfer of Se to grain. Therefore, further research on gene expression in nodes after Se applications should be explored.

Selenium applied by foliar application can enter the foliage through the epidermis or stomata, and then transported to the edible parts of plant (Luo et al., 2021). However, this study found that Se mainly remained in the leaves and sheaths after foliar Se application (**Figure 2**), although the Se content of wheat grains was significantly increased by 22–30% for Se application at pre-filling stage compared to pre-flowering stage. Further comparison of the TF of Se in different parts of wheat showed that the $TF_{rachis/first\ nodes}$ increased when exogenous Se was applied at pre-flowering stage compared with pre-filling stage (**Figure 3**). This result indicates that spraying exogenous Se at pre-filling stage increased the transfer of Se from the nodes to rachis, which shows that the efficiency of foliar Se application is higher at pre-filling stage. This observation is consistent with results obtained from field trials with wheat of Deng et al. (2017) and Wang et al. (2019).

Effects of exogenous selenium application on either selenium species distribution or selenium speciation variation

More than 50% of Se was stored in edible parts such as grains, beans, and leafy vegetables as organic Se, when different species of Se(VI) or Se(IV) were applied (Hart et al., 2011; Lavu et al., 2012; Poblaciones et al., 2014; Muleya et al., 2021). This study found that SeMet (87–96%) and $SeCys_2$ (4–13%) were the main Se species in wheat grains (93–100%; **Figure 6**), which is consistent with the findings of Poblaciones et al. (2014) and Hart et al. (2011). Similarly, Lu et al. (2018) showed that the main Se species of Se-enriched wheat was SeMet (44.2%), and $SeCys_2$ (2.6%) and MeSeCys (0.3%). Muleya et al. (2021) also found that corn can effectively convert inorganic Se into organic Se, and more than 92% of Se exists as organic forms. Regardless of the species of exogenous Se, organic Se is often the main Se species measured in Se-enriched mushrooms and peanuts (Zhou et al., 2019; Luo et al., 2021).

Se(VI) is difficult to be converted into organic Se compared with Se(IV; Mazej et al., 2008). Theoretically, the ratio of organic Se to total Se in wheat grains treated with Se(VI) should be lower than that in plants treated with Se(IV; Wang et al., 2020). However, no significant difference was found in the percentage of organic Se in wheat (grains) after applying different species of Se in this study. While Eiche et al. (2015) found that the major Se species were SeMeCys (about 70%) and SeCys (about 30%) in the grains of wheat grown in natural Se-enriched areas through XANES (X-ray absorption near-side structure).

In general, most of the exogenous Se was accumulated in wheat leaves after foliar Se application (Wang et al., 2020). The percentage of Se(VI) in wheat grains also increased with the higher Se application rate in foliar Se treatments (**Figure 6**), and foliar Se(VI) application at pre-filling stage. The percentage of Se(VI) increased by 4% compared with pre-flowering stage in wheat grains (**Figure 6**). During the grouting stage, the migration efficiency of organic Se into the wheat grains was higher than that of inorganic Se, indicating that there was a higher inorganic Se content in the outer layer of the grain (Carey et al., 2012). Based upon these reported data, an in-depth understanding of the formation of various parts of the grain, such as bran, endosperm, and germ, is critical to fully understand the distribution of Se in whole grains.

Effects of different flour yield on the bioaccessibility and content of Se in wheat

Recent studies have mainly focused on the bioaccessibility of Se in mushrooms (Zhou et al., 2019), grains and vegetables

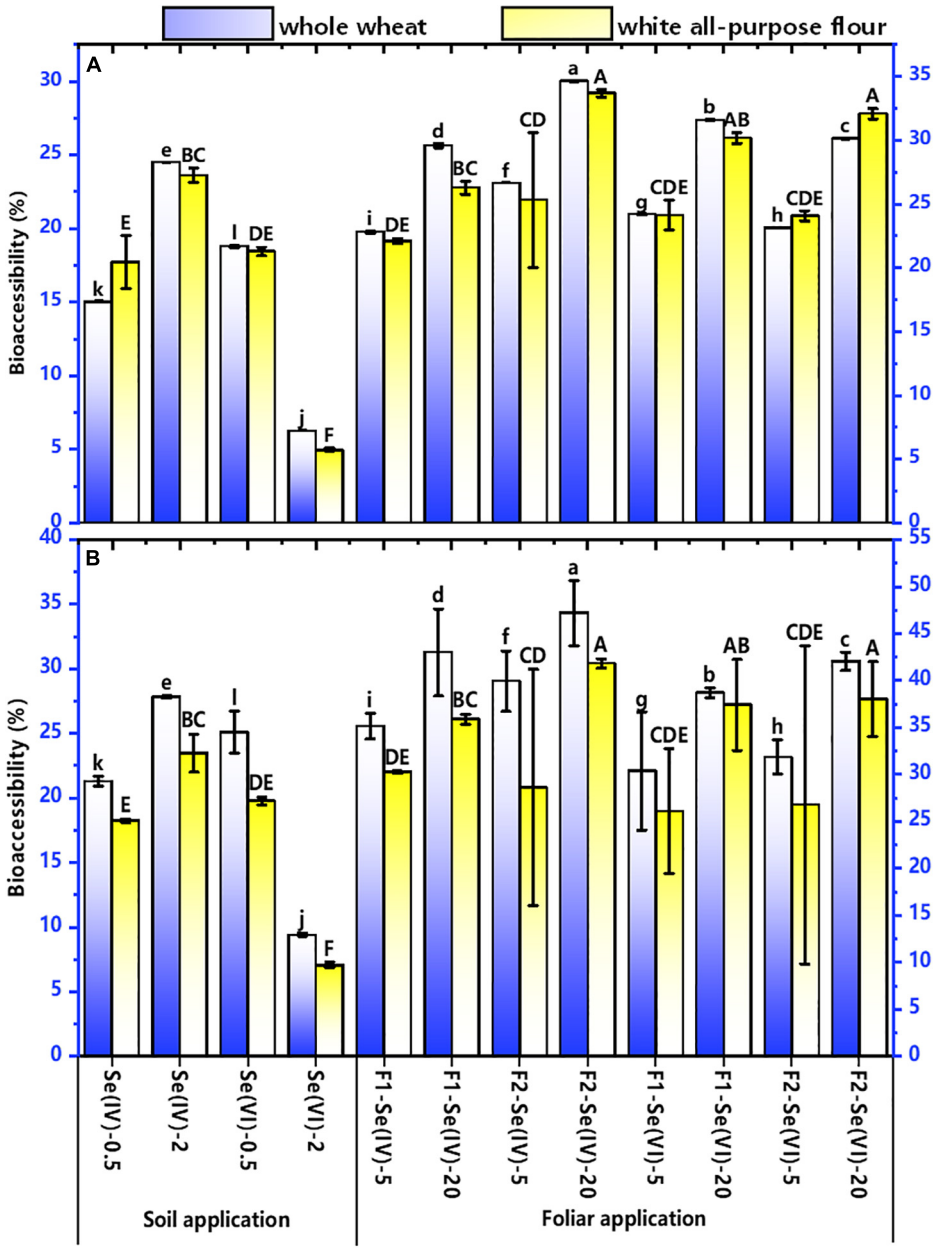

FIGURE 6
The bioaccessibility of Se in whole wheat and white all-purpose flour under different soil and foliar Se treatments. The bioaccessibility of Se in whole wheat and white all-purpose flour under different soil and foliar Se treatments. **(A)** represents the bioaccessibility of Se in gastric phase of whole wheat and white all-purpose flour with different Se treatments; **(B)** represents the bioaccessibility of Se in intestinal phase of whole wheat and white all-purpose flour with different Se treatments. Different lowercase letters of "a"–"l" indicate the significant ($p < 0.05$) differences between the bioaccessibility of Se in whole wheat with different Se treatments. Different uppercase letters of "A"–"F" indicate the significant ($p < 0.05$) differences between the bioaccessibility of Se in white all-purpose flour with different Se treatments.

(Zhou et al., 2020), lettuce (Do et al., 2017), radish (Hu et al., 2020), potato (Dong et al., 2020), and maize (Zhou et al., 2020). Studies showed that Se-enriched *Pleurotus ostreatus* and *Pleurotus florida* had high Se bioaccessibility, which reached 70–92% (Zhou et al., 2019) and 60–80%, respectively (Bhatia et al., 2013), while the Se bioaccessibility in cereals was low (corn, 51%; rice, 65%; Jaiswal et al., 2012). This study found that the bioaccessibility of Se in whole wheat and white all-purpose flour of different Se treatments ranged from 6 to 38%. These results are consistent with the findings of Khanam and Platel (2016), who found that the bioaccessibility of Se in wheat grains ranged from 10 to 24%. In addition, Zhou et al. (2020) also showed

that the Se bioaccessibility in maize was 8.8–22.5%. However, Lu et al. (2018) reported that the bioaccessibility of Se in Se-enriched wheat and soybeans reached 90%, corn and broccoli reached 80%, and cardamine hupingshanesis was 50%. These different percentages of Se bioaccessibility may have resulted from different Se application methods and types of crops.

Although the Se speciation in cereal crops has slightly different transformations (Muleya et al., 2021), no significant difference was found in Se bioaccessibility among them. The bioavailability of organic Se compounds is generally high (Muleya et al., 2021), due to the observation that organic Se is easily absorbed and utilized by humans (Gupta and Gupta, 2017). This study showed that organic Se was the main Se species in wheat grains (**Figure 6**). Consequently, the Se bioaccessibility in wheat should be higher. However, the observed low bioaccessibility of Se may be related to the bran component. The bioaccessibility of Se in white all-purpose flour was higher than that of whole wheat (**Figure 5**) confirmed that hypothesis. Reeves et al. (2007) found that the bioaccessibility of Se in refined wheat flour (mainly endosperm), wheat shorts (containing mainly germ), and wheat bran were 100, 85, and 60%, respectively. The low bioaccessibility of Se in bran is mainly because the Se-containing protein is wrapped by the non-digestible fiber in this component. Meanwhile, Khanam and Platel (2016) also found that the bioaccessibility of Se in intact legumes was lower than that of peeled legumes. Shen et al. (2019) showed that rice bran only accounts for about 7% of rice grain weight but contains about 14% of total Se in rice. Therefore, a considerable amount of Se may be lost in the process of removing wheat bran. Although the Se content of white all-purpose flour was lower than that of whole wheat, its bioaccessibility was high.

Different forms of applied exogenous Se also have different effects on the Se bioaccessibility of wheat. This study found that wheat treated with Se(VI) had higher Se bioaccessibility than Se(IV) treatments. Kápolna and Fodor (2007) also found that the bioaccessibility of Se in intestinal phase of Se-enriched green onions and leeks treated with Se (VI) was 80–90% and 12–28% with Se(IV) treatment. However, in the gastric phase of leek (*Allium ampeloprasum*), the Se bioaccessibility of Se(IV) treatment was slight higher than Se(VI) treatment (63 vs 56%), although this difference was not significant ($p > 0.05$; Lavu et al., 2012).

Regardless of the Se application methods, the Se bioaccessibility in intestinal phase of whole wheat and white all-purpose flour was higher than that in gastric phase. The results are consistent with the study of Lavu et al. (2012), which showed that the Se bioaccessibility in intestinal juice was 20% higher than gastric juice of Leek. The reasons may be as follows: (1) PBET is continuous (Toni et al., 2016), therefore, the Se bioaccessibility from the gastric phase to the intestinal phase is gradually accumulating; (2) in the intestinal phase, the existing digestive enzymes can hydrolyze polysaccharides, and then break down proteins into free amino acids and small molecular peptides, promoting the release of Se into the grains into the intestinal phase (Zhou et al., 2020). In this case, if a significant fraction of the bioaccessible Se has good chances to reach the colon, then it can be taken up by the microbial community and may also induce positive health effects. Further research is needed to evaluate whether this is actually the case.

Conclusion

This research is the first systematic study conducted to explore Se bioassessibility in wheat Se fortified with different Se application methods. The wheat was separated into nine parts (sheath, first internode, first node, and rachis haven't been systematically studied). The grain yield was the highest in plants treated with soil application at 2 mg kg^{-1} Se(IV), since Se(VI) has a higher Se bioavailability than Se(IV), there was an increased translocation of Se in wheat from the rachis to the grain. Both foliar and soil Se application can effectively increase the Se contents of wheat. The Se species applied to soil or to plant, application rates and growth stages applied, all influenced the Se content of wheat. Irrespective of Se application methods, the Se content of the first node was always higher than the first internode, indicating that the first node plays an important role in Se translocation in wheat. SeMet and $SeCys_2$ were the main Se species in grains of wheat, indicating that wheat can efficiently convert applied inorganic Se into organic Se within the plant. In addition, flour milling process will cause losses of Se in wheat. The percentages of lost Se in white all-purpose flour were 12–68% higher compared with whole wheat. The Se bioaccessibility of whole wheat and white all-purpose flour with different Se treatments ranged from 6 to 38%, and white all-purpose flour had higher Se bioaccessibility than whole wheat. Future studies should also focus on the speciation changes, genotypes, and influence of the nodes on the mechanisms of Se translocation within wheat.

Data availability statement

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

Author contributions

MW and DL created the hypothesis, objectives, outline the draft, and wrote the manuscript. FZ, NC, and PC performed the statistical analysis. YM, HZ, MQ, NL, YL, and LM performed the experiment. GB and DL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author NL was employed by Center of Regional Watershed Environment Comprehensive Control Technology in Jiangsu Province, Academy of Environmental Planning & Design, Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.988627/full#supplementary-material

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Selenium biofortification of soybean genotypes in a tropical soil *via* Se-enriched phosphate fertilizers

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Soybean is a major crop in Brazil and is usually grown in oxidic soils that need high rates of phosphate (P) fertilizers. Soybean is also very suitable for biofortification with Se, since its grains have high protein contents and are widely consumed worldwide (directly or indirectly). Few studies have addressed Se application under field conditions for soybean biofortification, especially in tropical soils. Here, we evaluated agronomic and physiological responses resulting from different strategies for biofortifying soybean grains with Se by applying this element *via* soil, using both conventional and enhanced-efficiency P fertilizers as Se carriers. The experiment was carried out at the Uva Farm, in Capão Bonito (São Paulo), Brazil. The experimental design was a randomized block split-plot design, with four fertilizer sources—conventional monoammonium phosphate (C-MAP), conventional monoammonium phosphate+Se (C-MAP+Se), enhanced-efficiency monoammonium phosphate (E-MAP), and enhanced-efficiency monoammonium phosphate+Se (E-MAP+Se), and four soybean genotypes (M5917, 58I60 LANÇA, TMG7061, and NA5909). The selenium rate applied *via* C-MAP+Se and E-MAP+Se was 80gha^{-1}. The application of the tested fertilizers was carried out at the sowing of the 2018/2019 cropping season, with their residual effect being also assessed in the 2019/2020 cropping season. Selenium application increased grain yield for the TMG7061 genotype. For all evaluated genotypes, Se content in grains increased in the 2018/2019 harvest with the application of Se *via* C-MAP+Se and E-MAP+Se. In general, the application of Se *via* C-MAP favored an increase in amino acid contents in grains and decreased lipid peroxidation. In summary, the application of Se-enriched P fertilizers *via* soil increased soybean grain yield, leading to better grain quality. No residual effects for biofortifying soybean grains were detected in a subsequent soybean cropping season.

KEYWORDS

biofortification, food security, cereal, nutritional quality, selenate

Introduction

Selenium (Se) is an essential element for humans and animals. It is a component of selenoaminoacids (e.g., selenocysteine), being necessary for the synthesis of more than 25 selenoproteins (Rayman, 2012; Oliver and Gregory, 2015). As a component of glutathione peroxidase, Se acts against oxidative stresses. In addition, Se also participates in thyroid metabolism and the immune system maintenance, reducing cancer and heart disease (Rayman, 2012; Avery and Hoffmann, 2018). It is estimated that about 1 billion people worldwide are Se deficient (Mora et al., 2015). Keshan and Kashin-Beck diseases are associated with Se deficiency in human organisms. Keshan is related to cardiomyopathy affecting children and young women and Keschin-Beck is related to osteoarthritis, promoting bone atrophy (Yao et al., 2011).

Selenium is not currently considered a plant nutrient though its beneficial effects on vegetables have been studied for over 70 years (Lyons et al., 2009; Feng et al., 2013). Several beneficial effects of this element for plants have been reported, such as improved rice growth (Boldrin et al., 2012), increased photosynthetic rate and wheat yield (Lara et al., 2019), reduced production of free radicals in lettuce (Ramos et al., 2011), increased protein content and total amino acids in soybean (Zhao et al., 2019), and reduced the damage caused by water stress in rice and common bean plants (Andrade et al., 2018; Ravello et al., 2021). For this reason, due to new trends in plant nutrient classification, Se and other beneficial elements (Na, Si, Al, Co, and I) may be considered plant nutrients in the future (Brown et al., 2021).

Selenium availability in soils depends on several factors, such as the Se source, soil mineralogy, redox condition, pH, and the presence of other anions (Lopes et al., 2017). Tropical soils are known for their high capacity to retain oxyanions—including selenite and selenate—with Se availability being decreased with increasing clay content. This is due to the high concentration of Fe/Al oxyhydroxides present in oxidic soils from tropical regions (Lopes et al., 2017; Araujo et al., 2018). Because of that, plants grown in soils with low Se concentration and availability show inadequate accumulation of this element in their edible parts (White and Broadley, 2009).

The adoption of biofortification practices is a suitable strategy to increase Se contents in food crops. Biofortification is a strategy that aims to increase the content of minerals and vitamins in crops *via* genetic (e.g., breeding) and/or agronomic (fertilization) practices (Cakmak, 2008; White and Broadley, 2009). Knowing the various constraints related to Se availability in Brazilian agroecosystems, the Brazilian Ministry of Agriculture, Livestock, and Supply approved a new legislation (normative N° 46/2016), which allowed the addition of Se in fertilizers marketed in Brazil (Brazil, 2016). A possible and relevant alternative to directly applying Se fertilizers in tropical agroecosystems could be its co-application *via* phosphate fertilizers, since the presence of competing anions, such as phosphate, reduces Se adsorption, increasing soil Se availability (Lessa et al., 2016; Mateus et al., 2021). Studies involving the biofortification of rice grown in tropical soils have reported the efficacy of the strategy of supplying Se to plants *via* its co-application with monoammonium phosphate—MAP (Lessa et al., 2020). Many P-fertilizer products are currently being used in oxidic soils with a technology to reduce phosphate retention (e.g., the so-called enhanced-efficiency products), it is thus relevant to determine if such technologies could improve Se use efficiency when selenium is soil-applied using enhanced-efficiency MAP as a carrier.

Additional studies evaluating Se application *via* soil associated with sources of phosphate fertilizers are still required. To the best of our knowledge, there are few studies in tropical soils assessing Se application, mainly focusing on the co-application of Se with phosphate fertilizers. Soybean is an interesting agricultural crop for biofortification with Se due to the large number of products generated from soybean grains, the high concentration of proteins, and the geographic distribution of soybean production. The present study aimed to evaluate the effectiveness of applying Se in association with phosphate fertilizers for soybean biofortification and its residual effect in the succeeding cropping season in tropical soils.

Materials and methods

Experimental area and treatments

The experiment was carried out with soybean crop (*Glycine max* L. Merril) grown under commercial field conditions during the cropping seasons of 2018/2019 (application of treatments with Se) and 2019/2020 (assessment of residual effects of Se previously applied) at the Uva Farm, located in Capão Bonito, State of São Paulo (SP), Brazil, at the following geographic coordinates: Lat: −24.040934, Lon: −48.262421 (Figure 1). The weather of the region is characterized as humid subtropical (Cfa), with an average rainfall of 1,628 mm and an average annual temperature of 18.8°C (Alvares et al., 2013).

The soil of the experimental region—Oxisol—is classified as Typic Hapludox (Soil Survey Staff, United States Department of Agriculture Natural Resources Conservation Service, 2014) and the chemical and physical properties are as follows, according to the methodology suggested by Brazilian Agricultural Research Company (EMBRAPA) (1997) [pH (H_2O) = 6.0; H + Al = 2.96; Al = 0.06; P (Mehlich-1) = 34.8 mg dm^{-3}; K = 148 mg dm^{-3}; S = 4.11 mg L^{-1}; CEC = 9.83 cmol$_c$ dm^{-3}; Ca = 5.05 cmol$_c$ dm^{-3}; Mg = 1.44 cmol$_c$ dm^{-3}; P-rem = 28.10 mg L^{-1}; organic matter = 2.69 dag dm^{-3}; clay = 510 g kg^{-1}; silt = 110 g kg^{-1}; and sand = 380 g kg^{-1}].

The experiment was arranged in a randomized block split-plot design, with four replicates. The biofortification of soybean was tested applying four different fertilizers: (i) Conventional monoammonium phosphate (C-MAP); (ii) Conventional monoammonium phosphate + Se (C-MAP + Se); (iii) Enhanced-efficiency monoammonium phosphate (E-MAP); and (iv)

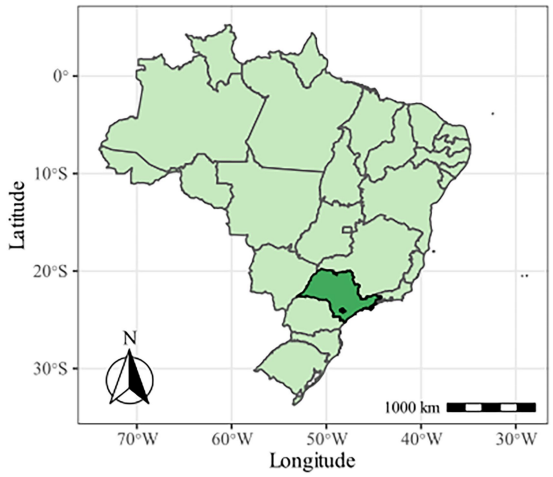

FIGURE 1
Location of the experimental area in Capão Bonito, SP, Brazil.

Enhanced-efficiency monoammonium phosphate + Se (E-MAP + Se). Monoammonium phosphate was coated with the humic and fulvic substances. The C-MAP + Se and E-MAP + Se fertilizers were prepared by spraying Se to the fertilizer granule. For this purpose, the fertilizers were coated after the granulation with 500 mg kg^{-1} of Se (from a solution of sodium selenate—Na_2SeO_4, Sigma-Aldrich, Saint Louis, MO, United States). Considering that 80 kg ha^{-1} of P_2O_5 were applied as MAP (~50% P_2O_5), the addition of Se-rich fertilizers (500 mg Se kg^{-1}) added a Se rate of 80 g ha^{-1}.

The aforementioned fertilizers were applied to four soybean genotypes, as follows: M5917 (maturity group = 5.9), 58I60 LANÇA (maturity group = 5.8), TMG7061 (maturity group = 6.1), and NA5909 (maturity group = 6.2; all of them presenting indeterminate growth type). Thus, the experiment had a total of 16 treatments, with four replicates, totaling 64 experimental plots. The fertilizers comprised the plots and the split-plots were represented by the genotypes. Each experimental split-plot was 30 m long by 3 m wide (soybean row spacing at 0.5 m, totaling 90 m^2). Planting was made with 14 seeds per meter and fertilization was carried out during the sowing at the soybean seeds line (localized placement) by applying 16 kg ha^{-1} of N, 80 kg ha^{-1} of P_2O_5, and 28 kg ha^{-1} of K_2O.

After the soybean harvest (described next), wheat was sown in the area but was not harvested for analysis. After wheat, soybean was sown in the succeeding summer crop to evaluate the residual effect of Se associated with the previously soil-applied phosphate fertilizer. Selenium treatments were not applied in this second season with all following the standard management carried out at the Uva farm.

Analysis of oxidative stress and antioxidant enzymes

The uppermost fully developed leaf (trifoliolate) from 10 plants during the first cropping season (2018/2019) were collected at the full pod stage (R4) to evaluate antioxidant enzymes and oxidative stress. The collected leaves were frozen immediately in liquid nitrogen and stored in a deep freezer at −80°C for subsequent analysis. After that, the frozen plant material (0.2 g) was macerated in a porcelain mortar with liquid nitrogen and polyvinylpolypyrrolidone (PVPP) and mixed with 1.5 ml of buffer solution (100 mM potassium phosphate at pH 7.8, 0.1 mM EDTA, and 10 mM ascorbic acid).

The extract was centrifuged at 13,000 *g* for 10 min at 4°C. The supernatant was collected for measuring the activities of the enzymes, as follows: superoxide dismutase (SOD; Giannopolitis and Ries, 1977), ascorbate peroxidase (APX; Nakano and Asada, 1981), and catalase (CAT; Havir and McHale, 1987). In addition to that, 0.3 g of macerated frozen material were homogenized with 1.5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 *g* for 15 min at 4°C for hydrogen peroxide (H_2O_2; Velikova et al., 2000) and peroxidation lipid (MDA; Buege and Aust, 1978).

Soil Se content

For the determination of total Se content (partially available) in the soil, one composite soil sample (coming from five subsamples distributed around the experimental plot) was collected in each experimental plot at the full pod stage (R4). The samples were dried, homogenized, ground with a mortar and agate pestle, and passed through a 100-mesh nylon sieve. A sample mass of 0.5 g was mixed with 5 ml of *aqua regia* (a mixture of HNO_3 65% and HCl 37%—1:3 v/v). The mixture/suspension was left to stand for 1 h, and the Teflon® vessels were hermetically sealed and heated in a Mars-5 microwave digestion oven (CEM Corp, Matthews, NC, United States) with a temperature set at 175°C and a controlled pressure of 0.76 MPa for 25 min. Next, the vessels were cooled to room temperature and the volume was completed to 40 ml with bidistilled water.

Selenium in the soil samples was analyzed by Graphite Furnace Atomic Absorption Spectrometry with Zeeman background correction and EDL lamp for Se (GFAAS; AAnalyst™ 800 AAS, Perkin Elmer). The calibration curve for Se measurements was obtained from a standard solution with 1 g L^{-1} of Se (≥98% of purity, Fluka, Buchs, Switzerland). The reference material used for soil Se concentration was SRM 2709a [San Joaquin Soil, from the National Institute of Standards & Technology (Gaithersburg, MD, United States)], which contains 1.5 mg kg^{-1} of Se. The mean recovery of Se in this certified material was 88%.

Harvest and yield determination

After R8 stage, when 95% of pods have attained maturity and have a variety-dependent color of brown or tan (134 and 495 days after the treatment application for the first and second

season, respectively), grains from the useful area of the experimental plot were harvested and weighed to determine crop yield. Grain moisture was measured using a portable meter (model G650i, Gehaka®) and grain yield was corrected to 13%. A sample of each harvested plot was ground in a Willey mill for the determination of Se, N, protein, and total free amino acids.

Nitrogen and selenium content in grains

Nitrogen quantification was performed by the Kjeldahl method described by Bremner (1996). The extraction for determination of Se was obtained by acid digestion of 0.5 g of ground grain, in a microwave oven, following the USEPA 3051A method (Usepa, 2007). Selenium contents were performed using an inductively coupled plasma mass spectrometer (ICP-MS; PerkinElmer, model NexIon 2000 B, Waltham, United States).

To ensure the quality of the digestion process, a reference standard from the Institute for Reference and Measurement Materials (White Clover – BCR 402, IRMM, Geel, Belgium, with 6.70 mg Se kg^{-1}) and a blank sample were added to each digestion batch. The detection limit (LOD) was obtained using Se measurement in seven blank extracts and was calculated from the Equation 1:

$$LOD = (x + t \times s) \times d \quad (1)$$

where:

x = mean content of the substance of interest in seven blank samples.

t = Student value to 0.01 of probability.

s = standard deviation.

d = dilution.

The fraction of the applied Se that was incorporated in soybean grains (Se recovery) was calculated using the Equation 2 described below:

$$\text{Se recovery}(\%) = \frac{(\text{Se treatment} - \text{Se control})}{\text{Se rate}} \times 100 \quad (2)$$

where:

Se recovery (%) = use efficiency of the Se rates applied in the soil by soybean grains (Se utilization percentage);

Se treatment (g ha^{-1}) = Se contents in soybean grains from soybean plants grown in treatments that received Se applications, considering the yield obtained in each treatment;

Se control (g ha^{-1}) = Se contents in soybean grains from soybean plants grown in treatments without Se applications, considering the yield obtained in each treatment; and

Se rate (g ha^{-1}) = Se rates applied in the soil.

Total free amino acids and protein

Total free amino acids were determined using the ninhydrin method (Yemm et al., 1954). The quantification of protein in the grains was determined by multiplying the value of the N content by 6.25.

Statistical analysis

The obtained data were primarily tested for their normality (Shapiro–Wilk's test) and homogeneity of variance (Bartlett's Test). Then, they were submitted to ANOVA, and when significant, mean values of variables found for each treatment were compared by the Tukey test at 5%. Principal component analysis (PCA) was performed for the dataset of conventional or enhanced fertilizer. The Pearson's linear correlation matrix ($p < 0.05$) was also carried out, aiming to validate clusters and potential relationships of Se application in soil and plant attributes as outcomes of PCA. The analyses were made using the R software (R Core Team, 2020).

Results

Soybean yield (cropping seasons of 2018/2019 and 2019/2020)

The tested factors (genotypes and fertilizer sources) affected soybean grain yield in the 2018/2019 season ($p < 0.05$). The fertilizer sources applied did not alter the yield of 58I60 LANÇA and M5917 genotypes. On the contrary, the genotype N5909 showed a statistical difference in yield by the Tukey's test ($p < 0.05$), between the application of C-MAP and E-MAP, with 92.08 and 76.36 bags ha^{-1}, respectively (Figure 2).

Grain yield in the TMG7061 genotype was higher in treatments using C-MAP + Se and E-MAP + Se when compared to C-MAP and E-MAP, reaching yields of 94.77 and 95.62 bags ha^{-1}, respectively and gains of 24.51 and 26.85 bags ha^{-1} in yield, respectively. In the 2019/2020 cropping season, when the residual effect of Se applied in the soil was evaluated, the factors tested did not affect grain yield ($p > 0.05$; Supplementary Table 1).

Selenium content in soybean grains and soil

Selenium content analyzed in the reference material was 7.37 mg kg^{-1}, indicating a recovery of 110%. The Se content in soybean harvested in the first season was influenced by the genotypes and sources of fertilizers applied ($p < 0.05$; Figure 3A). In all tested genotypes, the application of C-MAP + Se and E-MAP + Se increased the Se content in grains. In the genotype

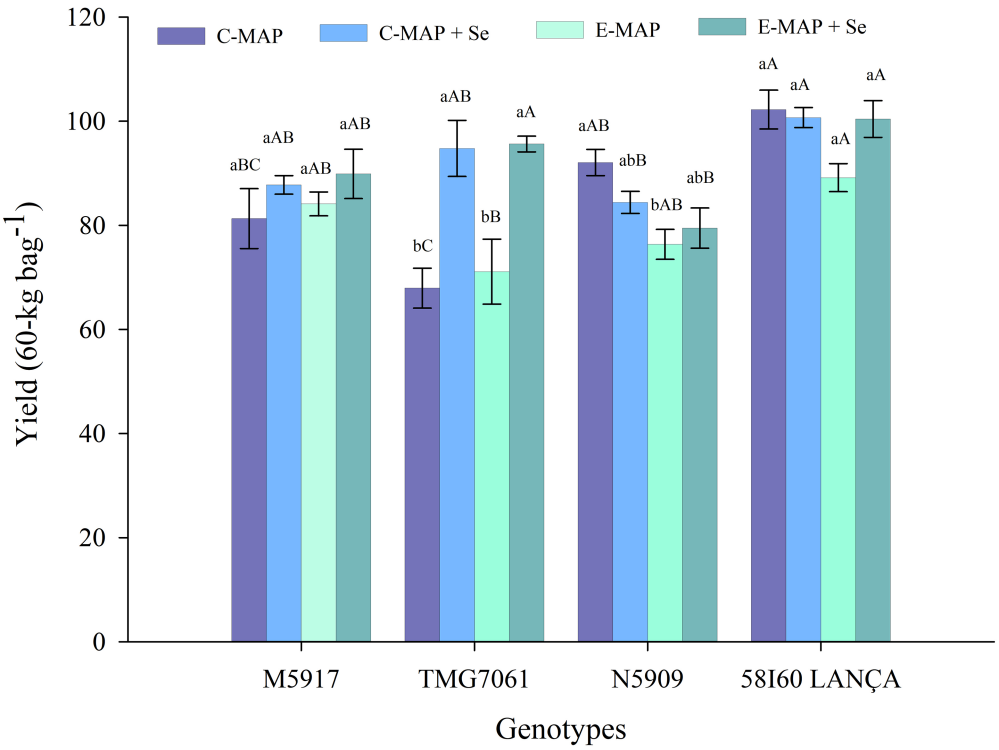

FIGURE 2
Yield (60-kg bags^{-1}) of soybean grains harvested from the 2018/2019 cropping season. Lowercase letters compare soybean yields among fertilizers in each genotype and capital letters compare soybean yields among genotypes in each fertilizer source at the level of 5% (p<0.05) by the Tukey test. The vertical bars refer to the standard error (n=4). C-MAP, conventional monoammonium phosphate; C-MAP+Se, conventional monoammonium phosphate+Se; E-MAP, enhanced-efficiency monoammonium phosphate; and E-MAP+Se, enhanced-efficiency monoammonium phosphate+Se.

TMG7061, the increase in Se content was 2.90 and 3.31 times greater with the application of C-MAP + Se and E-MAP + Se, compared with their respective fertilizers without Se. In the other genotypes, the application of C-MAP + Se and E-MAP + Se presented values higher than two times the Se content accumulated into grains when the fertilizers C-MAP and E-MAP were applied.

Observing the content of Se in grains, with the use of C-MAP + Se and E-MAP + Se, the genotype TMG7061 presented the highest content, being however statistically different only from the genotype 58I60 LANÇA for C-MAP + Se and from the genotype M5917 for E-MAP + Se. The Se recovery by soybean grains was different among the tested genotypes ($p < 0.05$; Figure 3B), with the genotype TMG7061 showing the highest value (close to 12.4%).

The Se content in soybean grains harvested in the 2019/2020 crop was not influenced by the variables analyzed ($p > 0.05$; Supplementary Table 1). The average grain contents as a function of the fertilizers applied were 0.48 mg kg^{-1} (E-MAP), 0.52 mg kg^{-1} (C-MAP), 0.55 mg kg^{-1} (E-MAP + Se), and 0.62 mg kg^{-1} (C-MAP + Se). In the soil, the Se content did not differ statistically among treatments. The overall average Se content found in the soil in phase R4 was 0.73 mg dm^{-3}, which justifies the low Se concentration in soybean grains of the crop carried out in the 2019/2020 cropping season (Supplementary Table 2).

Nitrogen, protein, and amino acids

Nitrogen content, proteins, and total free amino acids were affected by the interaction between the tested genotypes and fertilizers. Following the application of C-MAP, the genotypes M5917 and 58I60 LANÇA showed higher N and protein contents compared with other treatments (Figure 4A; Supplementary Table 1). The total free amino acid content was higher with C-MAP + Se than with the other fertilizer sources for genotypes N5909 and 58I60 LANÇA (Figure 4B). Total free amino acid contents did not change due to the fertilizer sources applied for genotype M5917, whereas for genotype TMG7061, the highest and lowest values were verified after the application of C-MAP + Se and E-MAP and E-MAP + Se, respectively.

Antioxidative metabolism

Overall, the activity of enzymes was not affected by the different fertilizers sources (Table 1). Superoxide dismutase and CAT had different activities among the genotypes, while APX was not affected by any of the factors under study. The genotype TMG7061 showed lower SOD activity and lower H_2O_2

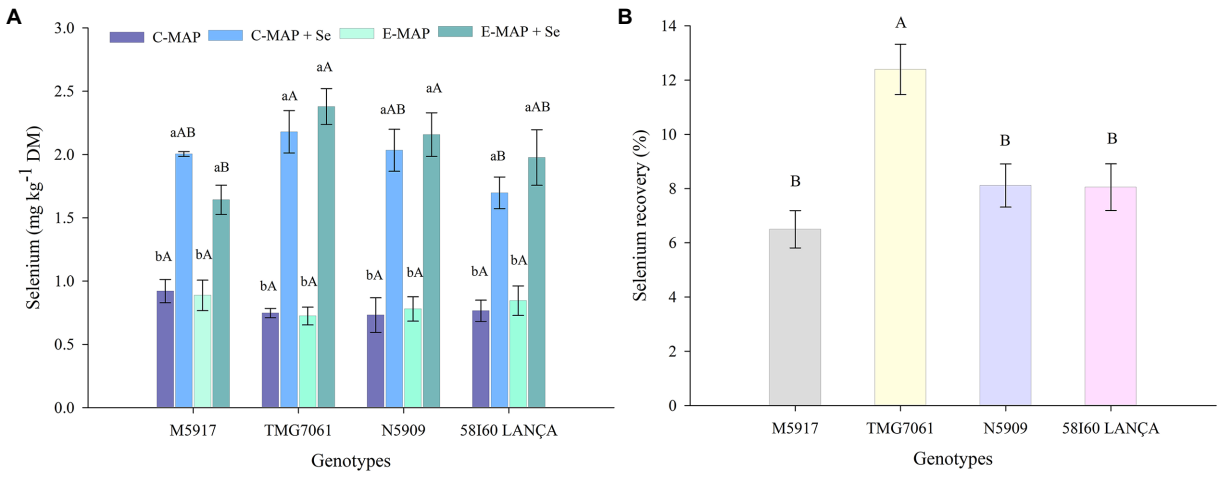

FIGURE 3
Selenium content (mgkg^{-1}) and Se recovery (%) **(B)** in soybean grains harvested from the 2018/2019 cropping season. Lowercase letters compare Se contents and Se recovery among fertilizers in each genotype and capital letters compare Se contents and Se recovery among genotypes in each fertilizer source at the level of 5% (p<0.05) by the Tukey test. The vertical bars refer to the standard error (n=4). C-MAP, conventional monoammonium phosphate; C-MAP+Se, conventional monoammonium phosphate+Se; E-MAP, enhanced-efficiency monoammonium phosphate; and E-MAP+Se, enhanced-efficiency monoammonium phosphate+Se. The vertical bars refer to the standard error (**A**—n=4; **B**—n=8).

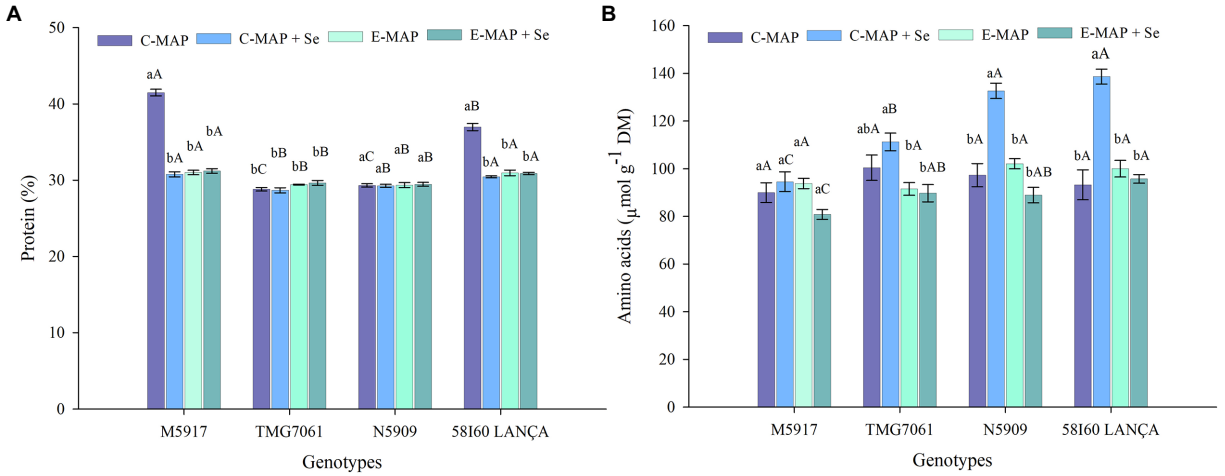

FIGURE 4
Protein (%) **(A)** and amino acids (µmolg^{-1} DM) **(B)** in soybean grains harvested from 2018/2019 cropping season. Lowercase letters compare protein and amino acids among fertilizers in each genotype and capital letters compare protein and amino acids among genotypes in each fertilizer source at the level of 5% (p<0.05) by the Tukey test. The vertical bars refer to the standard error (n=4). C-MAP, conventional monoammonium phosphate; C-MAP+Se, conventional monoammonium phosphate+Se; E-MAP, enhanced-efficiency monoammonium phosphate; and E-MAP+Se, enhanced-efficiency monoammonium phosphate+Se.

concentration. Among the sources of fertilizers applied, C-MAP presented higher H_2O_2 content (2.09 µmol H_2O_2 g^{-1} MF), yet it differed only from the treatment with the application of E-MAP + Se (1.54 µmol H_2O_2 g^{-1} MF).

Malonaldehyde (MDA) levels were affected by the interaction between genotypes and fertilizers (p < 0.05), with genotype TMG7061 being the only one that showed a difference among fertilizers. In this genotype, MDA levels were higher with the application of C-MAP, indicating an increase in lipid peroxidation.

Principal component analysis

With the application of the conventional MAP with and without Se (C-MAP and C-MAP + Se), 46.9% of the covariances were explained by the PC1 and PC2 axes (Figure 5A). For E-MAP and E-MAP + Se, 46.2% of the covariances were explained by the PC1 and PC2, but the confidence intervals overlapped (Figure 5B). For fertilizers C-MAP and C-MAP + Se, the PCA showed that the concentration of total free amino acids correlates positively with the application of Se. In addition,

TABLE 1 Effect of Se application *via* soil on the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), lipid peroxidation by the MDA, and hydrogen peroxide (H_2O_2) with SEs (n=4).

Genotype	Fertilizer	SOD (U SOD min^{-1} g^{-1} FM)	CAT (ηmol H_2O_2 min^{-1} g^{-1} FM)	APX (ηmol ASA min^{-1} g^{-1} FM)	MDA (ηmol MDA g^{-1} FM)	H_2O_2 (μmol H_2O_2 g^{-1} FM)
M5917	C-MAP	610.82 ± 18.66	2.97 ± 0.16	26.38 ± 2.01	15.94 ± 1.94 aAB	2.16 ± 0.23
TMG7061		616.14 ± 15.12	3.78 ± 0.47	29.37 ± 1.90	19.84 ± 0.58 aA	1.73 ± 0.27
N5909		647.83 ± 12.53	2.99 ± 0.30	23.55 ± 2.40	17.48 ± 1.45 aA	2.35 ± 0.09
58I60 LANÇA		658.31 ± 19.96	2.71 ± 0.29	23.36 ± 1.71	12.65 ± 1.31 aB	2.13 ± 0.24
M5917	C-MAP + Se	618.59 ± 31.55	3.36 ± 0.57	24.79 ± 2.29	13.40 ± 0.86 aA	2.00 ± 0.22
TMG7061		532.09 ± 25.74	3.37 ± 0.62	23.82 ± 2.49	13.48 ± 1.15 bA	1.35 ± 0.27
N5909		637.13 ± 13.51	2.23 ± 0.20	21.09 ± 2.50	13.08 ± 1.13 aA	1.87 ± 0.32
58I60 LANÇA		642.33 ± 24.15	1.59 ± 0.18	23.20 ± 2.87	13.94 ± 0.84 aA	1.89 ± 0.12
M5917	E-MAP	611.05 ± 24.87	2.46 ± 0.67	22.93 ± 4.04	11.77 ± 0.69 aA	2.10 ± 0.35
TMG7061		536.86 ± 21.09	3.35 ± 0.63	22.09 ± 4.12	12.45 ± 0.30 bA	1.13 ± 0.23
N5909		583.80 ± 21.11	2.58 ± 0.70	21.93 ± 2.08	15.71 ± 2.27 aA	1.88 ± 0.23
58I60 LANÇA		613.77 ± 26.17	2.69 ± 0.68	23.71 ± 3.31	14.25 ± 1.46 aA	2.11 ± 0.21
M5917	E-MAP + Se	619.08 ± 26.87	3.56 ± 0.47	28.84 ± 5.57	11.50 ± 1.42 aB	1.53 ± 0.20
TMG7061		546.71 ± 14.76	2.60 ± 0.57	21.29 ± 4.71	14.23 ± 0.59 bAB	0.97 ± 0.13
N5909		600.64 ± 18.22	2.78 ± 0.30	27.38 ± 4.84	12.86 ± 1.72 aAB	1.53 ± 0.29
58I60 LANÇA		639.01 ± 13.65	2.66 ± 0.77	26.23 ± 3.41	16.34 ± 1.93 aA	2.17 ± 0.28
M5917	General average to	614.88 ± 11.63 A	3.09 ± 0.25 AB	25.73 ± 1.77 ns	13.16 ± 0.75 ns	1.95 ± 0.13 A
TMG7061	genotypes	557.95 ± 12.44 B	3.27 ± 0.28 A	24.14 ± 1.34 ns	15.00 ± 0.81 ns	1.29 ± 0.13 B
N5909		617.35 ± 10.06 A	2.65 ± 0.20 AB	23.49 ± 1.54 ns	14.78 ± 0.91 ns	1.91 ± 0.13 A
58I60 LANÇA		638.36 ± 10.47 A	1.09 ± 0.27 B	24.13 ± 1.34 ns	14.30 ± 0.73 ns	2.08 ± 0.10 A
C-MAP	General average to	633.27 ± 16.57 ns	3.11 ± 0.30 ns	25.66 ± 2.00 ns	16.48 ± 1.32 ns	2.09 ± 0.21 a
C-MAP + Se	fertilizers	607.54 ± 23.74 ns	2.64 ± 0.39 ns	23.23 ± 2.54 ns	13.47 ± 0.99 ns	1.78 ± 0.23 ab
E-MAP		586.37 ± 23.31 ns	2.77 ± 0.67 ns	22.67 ± 3.39 ns	13.55 ± 1.18 ns	1.81 ± 0.26 ab
E-MAP + Se		601.36 ± 18.37 ns	2.90 ± 0.53 ns	25.93 ± 4.63 ns	13.73 ± 1.42 ns	1.55 ± 0.22 b

Lowercase letters compare among fertilizers in each genotype and capital letters compare among genotypes in each fertilizer source at the level of 5% ($p < 0.05$) by the Tukey test. No significance analysis was performed for SOD, CAT, APX, and H_2O_2 within soybean genotypes, as this was not the purpose of this study. C-MAP, conventional monoammonium phosphate; C-MAP + Se, conventional monoammonium phosphate + Se; E-MAP, enhanced-efficiency monoammonium phosphate; and E-MAP + Se: enhanced-efficiency monoammonium phosphate + Se. ns, no significant.

the soybean grain yield from the cropping season of 2018/2019 was favored by Se application. The significance of the correlation among the studied variables was confirmed by Pearson's linear correlation matrix ($p < 0.05$; Supplementary Figure 1).

Discussion

Yield

The average yield found in this study (89.7 bags ha^{-1}) was above the national average (50.0 bags ha^{-1}; Conab, 2022). This high average yield is related to the management adopted by the Uva farm and to the high soil fertility, based on soil attributes and nutrient concentration (e.g., P and K). To establish homogeneity in the final stand of plants and because all field operations were performed using commercial planting machines, the number of seeds that were sown per linear meter was the same for all genotypes, even though a higher number of seeds per linear meter was recommended for genotype TMG7061. Due to the presence of a larger stand of plants for this genotype (TMG7061), lodging of the plants occurred during the grain filling stage. Under high planting density, the light capture is reduced, reducing photosynthetic activity and carbohydrate accumulation in the stem, which leads to lodging (Song et al., 2020).

In addition to the high average yield, Se application increased grain yield for the TMG7061 genotype (Figure 2). The response of Se application to plant yield may vary depending on the genotype used (Thavarajah et al., 2015; Liu et al., 2021; Sher et al., 2022). At present, there are still very few specific reports on Se application in the soybean yield. In the principal component analysis, this increase in yield, correlated better with Se in the grains of soybean, when the plant was grown in soil fertilized with C-MAP + Se fertilizer (Figure 5A). In the work carried out by Deng et al. (2021), soil Se application also increased soybean yield compared with a control treatment. Previous studies have shown that Se can improve growth and increase antioxidant capacity in plants, which can affect yield, mainly when plants are exposed to stress factors (Boldrin et al., 2013; Nawaz et al., 2015; Mateus et al., 2021; Ravello et al., 2021).

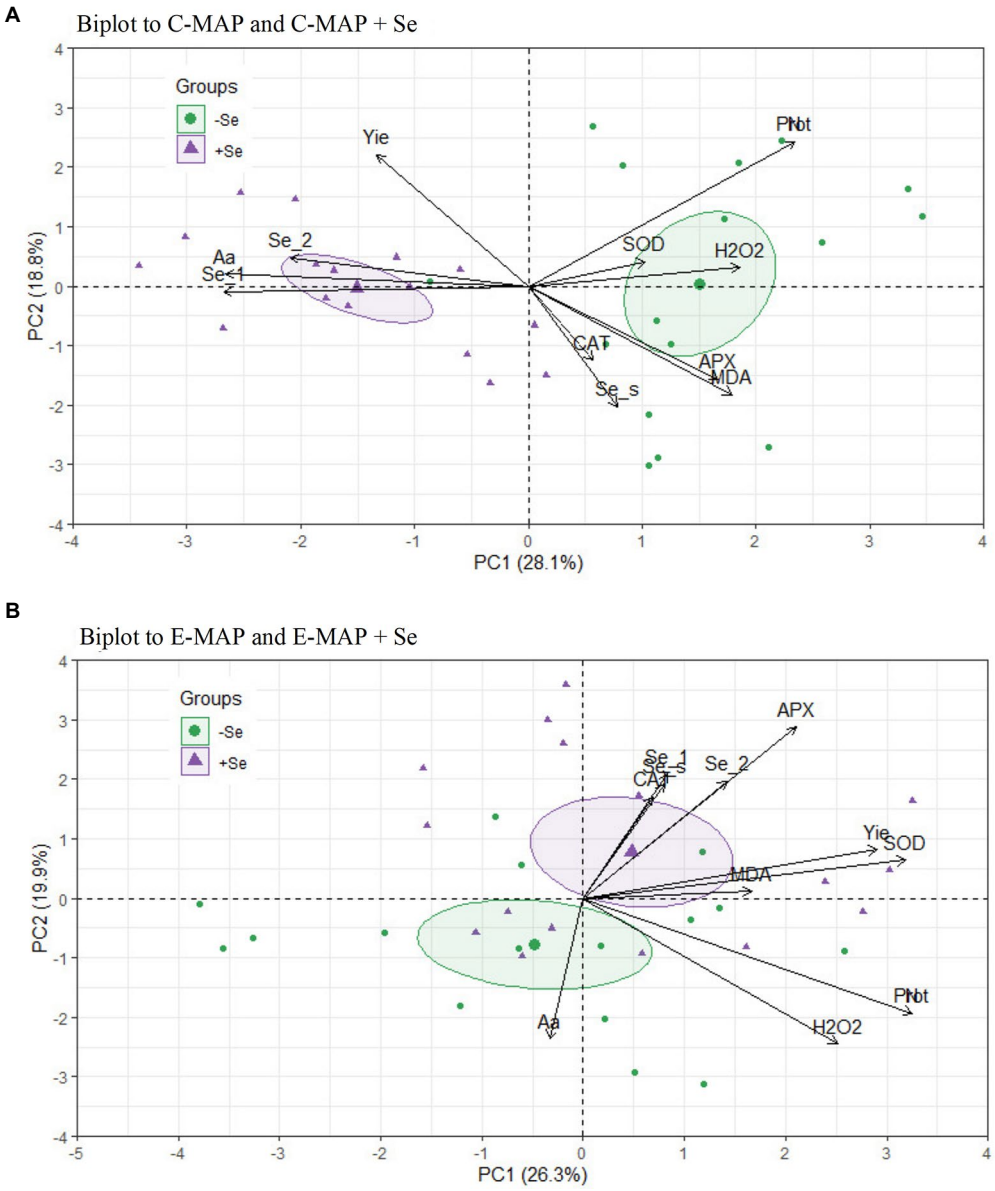

FIGURE 5
Biplot of principal component analysis (PCA) separated according to the fertilizers, **(A)** C-MAP and C-MAP + Se and **(B)** E-MAP and E-MAP + Se. Se content in grains of the cropping season of 2018/2019 (Se_1), Se content in grain of the cropping season of 2019/2020 (Se_2), Se in soil (Se_s), yield (Yie), protein in grains (Prot), amino acids in grains (Aa), lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX).

Enzymes

It has previously been established that Se can mitigate oxidative stress due to ROS regulation. This regulation can occur by stimulating the dismutation of O^{2-} into H_2O_2, by the regulation of enzymatic and non-enzymatic compounds, by the direct elimination of ROS by Se species, and by regulation of photosynthetic compounds (Silva et al., 2020). With Se application *via* C-MAP + Se, the MDA production was negatively correlated with Se content in grains, i.e., the production of MDA by leaves was lower as the Se content in grains increased (Supplementary Figure 1A). This reduction in MDA production demonstrates a clear ability to control ROS and thus oxidative stress, maintaining the integrity of cell membranes, allowing the maintenance of photosynthetic and productive performance of the plant, in addition to increasing Se contents in grains.

The activity of SOD and CAT enzymes was not influenced by the Se application, yet the formation of hydrogen peroxide was higher with the application of C-MAP, compared with E-MAP + Se in all genotypes (Table 1). In addition, the genotype TMG7061 was more sensitive to this change than the others, resulting in higher production of MDA when C-MAP

was applied. However, the higher production of hydrogen peroxide acted as a priming/beneficial stress effect, allowing the plant to adjust for grain yield, not exceeding its limit of physiological plasticity capacity, which could lead to a decrease in productivity (Agathokleous et al., 2020). According to the PCA and the Pearson's correlation matrix, ascorbate peroxidase activity in plants treated with Se application *via* E-MAP is positively correlated with Se content in grains. Lessa et al. (2020) showed that CAT, SOD, and APX activity had minimal interference from Se application *via* soil or leaf in rice, at a dose of 80 g ha^{-1}.

According to Djanaguiraman et al. (2005), Se foliar application to soybean (50 ppm) increased the activity of SOD, glutathione peroxidase (GSH-Px), and proline, causing a decrease in lipid peroxidation and the reduction in plant senescence. The activity of stress mitigation enzymes, such as SOD, is increased under conditions with high ROS production. Moreover, with adequate levels of Se, the enzyme GSH-Px acts on the spontaneous reduction of $O^{\cdot 2-}$ (Hartikainen et al., 2000; Feng et al., 2013).

Nutritional quality of grains and Se content in the soil

The average Se content found in the studied soil (0.73 mg dm^{-3}) is within the range of Se contents reported for soils of the State of São Paulo (where the Uva farm is located), which varies from <0.08–1.61 mg dm^{-3}. Soil Se content is influenced by characteristics such as pH (Schiavon et al., 2020), presence of competing ions such as sulfate and phosphate (Lessa et al., 2016; Santos et al., 2022), soil texture (Araujo et al., 2018), organic matter (Li et al., 2017), and presence of microorganisms (Gregorio et al., 2006).

Selenium content in grains harvested in the first crop season was higher in all genotypes with Se application, either *via* C-MAP + Se or *via* E-MAP + Se (Figure 3A). Considering the daily soybean intake of 50 g per person and the concentration of 2.37 mg kg^{-1} of Se in grains with the application of E-MAP + Se in the N5909 genotype, the concentration of Se ingested would be 118.5 µg day^{-1}, a value that lies above the average daily intake of Se recommended for adults (70 µg day^{-1}; Kipp et al., 2015).

The consumption of soybean by humans, for the most part, occurs indirectly as in the case of soybean sauce. The production of soybean sauce using biofortified soybean with Se is an alternative to increasing the Se intake by population utilizing supplementation of dietary change. Indeed, soybean sauce represents a strong antioxidant system, which keeps Se stable and non-toxic during storage (Gao et al., 2019, 2022). A study carried out by Gao et al. (2022) showed that soybean sauce produced from soybeans containing 259 µg kg^{-1} of Se contains 79.2 µg kg^{-1} of Se, with 24.8% being inorganic Se and 75.2% existing as organic Se form. This suggests that it is possible to produce a biofortified sauce using Se-enriched soybeans in the field.

The Se recovery observed in soybean ranged from 6.49 to 9.74% (Figure 3B). These values were higher than those reported by Lessa et al. (2020), who worked with soil Se fertilization in rice (maximum recovery = 2.7) and by Lara et al. (2019), who studied foliar application of Se in wheat (maximum recovery = 3%). This higher Se recovery by soybean grains can be attributed to its high protein concentration (about 40%). In the plant, sulfur present in selected amino acids can be replaced by Se, forming selenoaminoacids, which later form selenoproteins (White, 2016). Chan et al. (2010) found that selenospecies - including SeCys and SeMet - represent about 74% of the Se total in soybean grains, when this crop was treated with sodium selenite. Again, such results reinforce that soybean is an effective species when considering the biofortification of crops with Se.

Another factor that may have contributed to the greater Se recovery in soybean is the Se application associated with phosphate fertilizer. According to Qingyun et al. (2016), soils with nutrient deficiencies, especially P, may lead to reduced accumulation of Se in grains by crops. Phosphorus in soils occurs in anionic forms, which means that Se (as selenite—$NaSeO_4^-$) might compete with phosphate molecules for adsorption sites. However, the rates of phosphate fertilizers are much higher (nearly three orders of magnitude) than the amount of Se applied in this trial, making the retention of P more likely in these soils instead of the retention of Se.

In tropical soils, this competition between phosphate and selenite as well as between selenate and sulfate due to chemical similarities between them is acknowledged in the literature (Lessa et al., 2016; Lopes et al., 2017). The selenate adsorption process occurs mainly *via* formation of outer-sphere complexes, i.e., thru non-specific adsorption. However, for selenite, the formation of inner-sphere complexes occurs with the exchange of ligands, as well as phosphate, which for the most part is irreversible (McBride, 1994).

In the 2019/2020 cropping season, Se content in grains was lower (0.54 mg kg^{-1}) than the first season, and there was no difference among treatments (Supplementary Table 1). This shows that there is a low residual effect of the soil-applied Se in the 2018/2019 season, irrespectively of the fertilizer applied, mainly after the cultivation of a winter crop (wheat). The low residual effect can be confirmed by the low Se concentration found in the soil in the R4 development phase (soil sampling time) during the first crop season. Indeed, studies have reported that part of the soil-applied Se can be fixed within a few months after application, making it unavailable for plant uptake (Gissel-Nielsen and Bisbjerg, 1970; Mikkelsen et al., 1989), which might be especially relevant for the case of the oxidic soil used in this study.

When applied as selenate, Se is found to be more available in soils than selenite in the short term. However, over time, Se^{VI} can be reduced to lower valence state species (e.g., Se^{IV}), leading to further adsorption of the reduced species onto surfaces, including Fe/Mn/Al oxides. This effect occurs faster in acidic soils than in alkaline soils (Wang et al., 2017). Indeed, Ramkissoon et al. (2021) have reported that when selenate was applied in an Oxisol (pH = 6.8 and clay = 52%), 75% was adsorbed during the first day, which impaired the quantification of soluble Se 300 days after the application. The authors presumed that the oxides present in soil

were responsible for Se sorption in this case. By contrast, soluble Se have decreased only 29% on a calcareous soil (pH = 8.2 and clay = 13%) after 300 days (Ramkissoon et al., 2021). This fact supports our findings, indicating that the low residual effect of Se at the second season is most likely related to selenium adsorption by soil.

In soils with low Se concentration (e.g., tropical regions), Se supply *via* fertilization is essential for biofortification strategies, especially in areas with no or low Se addition. However, the beneficial effects of fertilizer Se carried out in one season does not persist and that successive applications, associated with the application of other oxyanions that can compete with Se for oxidic sorption sites (e.g., phosphate and sulfate) as well as the addition of organic compounds *via* soil tend to increase the residual effect of Se in the soil (Qingyun et al., 2016). Indeed, the application of NPK fertilizer, associated or not with organic compost, has been reported to increase Se availability by 38.39 and 33.04% over 20 years (Qingyun et al., 2016).

The amount of total protein in soybeans was not increased by Se treatment. This fact supports the findings made by Yang et al. (2003) and Deng et al. (2022). However, the application of C-MAP + Se increased the free total amino acid content in genotypes N5909, Lança, and TMG7061. The results were consistent with previous studies indicating that an increase of Se in the crop could promote amino acids synthesis and thus improve amino acid content of Se-enriched soybean grains (Zhao et al., 2019).

Conclusion

This present study showed that the application of C-MAP + Se and E-MAP + Se fertilizers is a promising method for biofortifying soybean with Se in tropical soils. This fact was especially relevant in the TMG7061 genotype when, the application of these fertilizers increases crop yield. In addition, the TMG7061 genotype showed greater recovery of Se by the grains. In summary, soybean is a good crop to be used in biofortification programs due to its high protein content and high capacity of Se recovery by the grains. Lastly, it is noteworthy the positive effect of the application of C-MAP + Se in grain quality, as it not only increased Se but also the amino acids content in the grains.

Data availability statement

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

Author contributions

MAS: conceptualization, resources, writing—original draft, and writing—review and editing. GFS: conceptualization, resources, and writing—review and editing. APBC, JLL, and GSD: resources and writing—review and editing. CO: writing—review and editing. GL, DA, and PB: conceptualization and writing—review and editing. LRGG: conceptualization, funding acquisition, resources, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

GSD was employed by ICL South American.

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

GSD declares that this study received support from ICL South American. The funder had the following involvement in the study: providing support for travel expenses to/from the field experiments and helping with sample collection.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.988140/full#supplementary-material

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Prediction models for monitoring selenium and its associated heavy-metal accumulation in four kinds of agro-foods in seleniferous area

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Se-rich agro-foods are effective Se supplements for Se-deficient people, but the associated metals have potential risks to human health. Factors affecting the accumulation of Se and its associated metals in Se-rich agro-foods were obscure, and the prediction models for the accumulation of Se and its associated metals have not been established. In this study, 661 samples of Se-rich rice, garlic, black fungus, and eggs, four typical Se-rich agro-foods in China, and soil, matrix, feed, irrigation, and feeding water were collected and analyzed. The major associated metal for Se-rich rice and garlic was Cd, and that for Se-rich black fungus and egg was Cr. Se and its associated metal contents in Se-rich agro-foods were positively correlated with Se and metal contents in soil, matrix, feed, and matrix organic contents. The Se and Cd contents in Se-rich rice grain and garlic were positively and negatively correlated with soil pH, respectively. Eight models for predicting the content of Se and its main associated metals in Se-rich rice, garlic, black fungus, and eggs were established by multiple linear regression. The accuracy of the constructed models was further validated with blind samples. In summary, this study revealed the main associated metals, factors, and prediction models for Se and metal accumulation in four kinds of Se-rich agro-foods, thus helpful in producing high-quality and healthy Se-rich.

KEYWORDS

Se-rich agro-food, selenium, metals, metals accumulation, prediction model

Introduction

Selenium (Se) has been listed as one of the essential micronutrients by the World Health Organization and the International Health Organization in 1973 (1, 2). Approximately one billion people in the world were estimated to be deficient in Se (3). Severe Se deficiency in the human body could cause myocardial failure, Keshan disease, and Kashin-Beck disease (4–6). China is one of the countries with severe Se deficiency in the world. Se could not be produced by the human body itself; it could only be obtained through diet or pharmaceuticals (7). Qualified Se-rich agro-foods have the characteristics of "safety, high quality, and health," and they could provide a convenient method to replenish Se in Se-deficient people (8). For example, people in Se-deficient areas, such as Finland, the United Kingdom, Australia, and New Zealand, have used Se-rich agricultural products to increase dietary Se intake to meet the daily Se needs of the human body (9–11).

However, the distribution of Se in nature is uneven and generally exists in a dispersed state. Se often coexists with metals, forming Se-Hg ore, Se-Cu ore, Se-Pb ore, etc., resulting in Se in the natural environment often associated with heavy metals such as Cd, Hg, and Pb (12–14). Moreover, with the development of industrialization and urbanization, sewage irrigation, agricultural materials (such as chemical fertilizers, pesticides, and plastic film) application and solid waste stacking are more serious than before, which may also cause heavy metal pollution in soil (15, 16). Considering the association relationship between Se and heavy metals, the soil heavy metal pollution in Se-rich areas is normally more serious than that in non-Se-rich areas. Thus, agro-foods grown in Se-rich areas could enrich not only Se but also heavy metals, which makes heavy-metal assessment of the Se-rich soil essential before crops planting (17).

Rice is a staple food for residents in many areas of China, and its ability to accumulate Se is strong (18). Therefore, Se-rich rice is regarded as a good Se supplement (19). However, previous studies have shown that rice could easily absorb and accumulate heavy metals from soil (20, 21). Li et al. (22) reported that the Cd and Cd were the dominant contaminants in rice grain, and their concentration were highly influenced by the soil pH, soil organic matter (SOM), Fe fraction and cultivar. Considering the existed association phenomenon of Se and metals in Se-rich rice, the intake of Se-rich rice could become a method of exposure to heavy metals for people who are supplementing Se through diet (23). Besides, many other Se-rich agro-products, such as garlic, mushroom, egg, and meat, are threatened by heavy-metal pollution (7). Therefore, screening factors affecting the accumulation of Se and heavy metals and studying the prediction models of accumulation of Se and associated metals in Se-rich agro-foods are of great importance for controlling the transmission of heavy metals in the food chain and accurately assessing human health risks.

Many prediction models of heavy metals in common agro-foods were established on the basis of indoor simulation experiments or in soil–crop systems (24–27). Most of these models predicted heavy metal content in crops with heavy-metal content in soil and the physical and chemical properties of soil (28, 29). For example, Xu et al. (30) constructed the prediction model of Pb accumulation in rice grain, found that factors containing content of total Pb, clay and SOM performed well in predicting the Pb content in grain. The migration of heavy metals in the soil–crop system not only depends on the content of heavy metals in soil but also the soil physical and chemical properties, which affect the distribution of heavy metals in soil and control the solid-liquid phase distribution of heavy metals, thus affecting the absorption of heavy metals by crops (26, 31). Regression models of loquat trace element concentrations showed that under specific soil condition, the accumulation of Cd, As, and Pb of loquat fruit were controlled by the Ca concentration, metal fraction and Fe content in soil, respectively (32). Similarly, according to the multiple linear regression models constructed by Shi et al. (33), the Pb accumulation in pepper could be quantitatively predicted by soil Pb content, pH and soil cation exchange capacity. Therefore, to improve the accuracy of the prediction results when predicting Se and heavy metal contents in Se-rich agro-foods, metal contents in soil, and soil properties, such as pH and the content of SOM, could be considered as evaluation indicators. At present, the prediction models for the distribution of Se and associated metals in Se-rich agro-foods have not been reported.

In this study, representative main Se-rich producing areas in China were selected to investigate and collect Se-rich rice, eggs, black fungus, and garlic samples and Se-rich soil, matrix, feed, irrigation, and feeding water samples. This study aimed to analyze the distribution of Se and its associated metals in agro-foods, soil, matrix, feed, and water; to screen the main types of associated metals present in Se-rich rice, eggs, black fungus, and garlic in China; and to establish and verify the prediction of Se and its associated metals in these Se-rich agro-foods.

Materials and methods

Sample collection

Selenium-rich rice sample collection

The collection of samples associated with Se-rich rice has been reported in the author's previous work (23). In total, 182 samples of Se-rich rice seeds, Se-rich soil, and water were collected, with at least 1 kg for each sample.

Selenium-rich garlic sample collection

Selenium-rich garlic, soil, and water used for Se-rich garlic cultivation were collected at the garlic maturity period from May to December in 2019. The sampling sites are distributed in five

representative Se-rich garlic production areas (Jinan, Taoyuan, Hailun, Haidong, and Enshi) in China. At least three large-scale Se-rich garlic-producing bases at each area were selected for sample collection, and at least five set of samples (Se-rich garlic, soil, and irrigation water) were collected at each base. In total, 157 samples of Se-rich garlic, Se-rich soil, and water were collected, with at least 1 kg for each sample.

Selenium-rich black fungus sample collection

Selenium-rich black fungus and the matrix and water used for Se-rich black fungus cultivation were collected from May to November in 2019. The sampling sites are distributed in five representative Se-rich black fungus production areas (Ankang, Shitai, Fengcheng, Jiamusi, and Enshi) in China. At least three large-scale Se-rich black fungus-producing bases at each area were selected for sample collection, and at least five set of samples (Se-rich black fungus, matrix, and water) were collected at each base. In total, 193 samples of Se-rich black fungus, Se-rich matrix, and water were collected, with at least 1 kg for each sample.

Selenium-rich egg sample collection

Selenium-rich eggs, the feed and water used for Se-rich egg production were collected from May to October in 2019. The sampling sites are distributed in five representative Se-rich egg production areas (Ankang, Fengcheng, Hailun, Zibo, and Enshi) in China. At least three large-scale Se-rich egg-producing bases at each area were selected for sample collection, and at least five set of samples (Se-rich egg, feed, and water) were collected at each base. In total, 129 samples of Se-rich eggs, Se-rich feed, and water were collected, with at least 1 kg for each sample. Figure 1 presents the sampling sites.

Sample pretreatment

The collected rice seeds were washed with deionized water and dried in an oven at 60–70°C until reaching a constant weight. The seeds were dehulled to obtain rice grain and then grounded into fine powder. The collected garlics were washed with deionized water and broken into vegetable puree with liquid nitrogen. The dried black fungus was grounded into fine powder. The eggs were washed with deionized water. The egg samples were stored at 4°C, and the rice, garlic, and black fungus samples were stored at −20°C until they were used for further analysis.

Stones and plant tissues were removed from the collected soil samples. The soil was smashed and passed through a 40-mesh sieve after air drying. Then, the sieved soil was grounded and passed through a 100-mesh sieve. The matrix and feed samples were grounded and passed through a 20-mesh sieve after air-drying. The sieved samples of matrix and feed were then passed through a 100-mesh sieve after grounding again. The powder of soil, matrix, and feed was stored in a dry environment at room temperature and used for further analysis.

The collected water, including the irrigation water and feeding water, was filtered with a 0.45 μm filter membrane and injected into the sampling bottle. HNO_3 was added to the sampling bottle to acidify the water (pH < 2) to fix the heavy-metal elements in the water sample. The pretreated water was stored at 4°C and used for further analysis.

FIGURE 1
Distribution of sampling sites.

TABLE 1 PRIs and ULs of selenium and PTDIs and maximum residue limit in agro-foods of potential associated metals.

Elements	PRI (μg/day)			UL (μg/day)			PTDI (μg/kg bw/day)	Maximum residue limit in agro-foods(mg/kg)		
	China	WTO/ FAO	EFSA	China	WTO/ FAO	EFSA	JECFA	China	EU	CAC
Se	60	34 men 26 women	70	400	–	300	–	–	–	–
Zn	12500 men 7500 women	3000–9800	7500–12700	40000	14000	16300	1000	–	–	–
Cd	–	–	–	–	–	–	0.83 (25 μg/kg bw/month)	0.2 rice 0.1 garlic 0.5 black fungus 0.05 egg	0.2 rice 0.1 garlic 1.0 black fungus	0.4 rice 0.05 garlic
Cr	30		–	–	–	–	0.957 (6.7 μg/kg·bw/ month)	1.0 rice 0.5 garlic	–	–
As	–	–	–	–	–	–	Not possible to establish a new PTWI that would be considered health protective	0.2 rice 0.5 garlic 0.5 black fungus	–	0.2 rice
Pb	–	–	–	–	–	–	Not possible to establish a new PTWI that would be considered health protective	0.2 rice 0.1 garlic 1.0 black fungus 0.2 egg	0.2 rice 0.1 garlic	0.2 rice 0.1 garlic
Hg		–	–	–	–	–	4	0.02 rice 0.01 garlic 0.1 black fungus 0.05 egg	–	–

PRI, population reference intake; UL, tolerable upper intake levels; PTDI, provisional tolerated daily intake.

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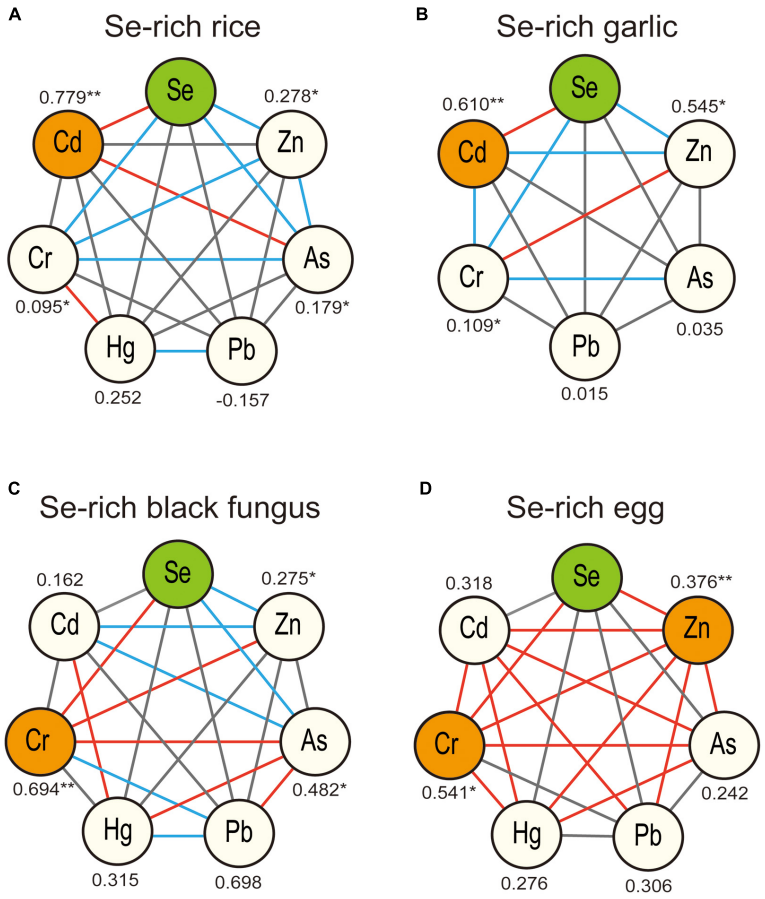

FIGURE 2
Pearson correlations analysis of Se and potential coexisting elements in Se-rich rice **(A)**, garlic **(B)**, black fungus **(C)**, and egg **(D)**. **Represents for highly significant correlation, $p < 0.01$, *represents for significant correlation, $p < 0.05$. The connections between the nodes represent for the significance of the correlation between different elements, solid red line represents for highly significant correlation, $p < 0.01$, solid blue line represents for significant correlation, $p < 0.05$.

Sample analysis

Analysis of contents of selenium and heavy metals

The contents of total Se and its associated metals, including Cr, Hg, Zn, Cd, Pb, and As, in all samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The population reference intakes (PRIs), tolerable upper intake levels (ULs), provisional tolerated daily intakes (PTDIs) and maximum residue limit values of these elements are listed in **Table 1**. The detailed procedures and parameters for microwave digestion and ICP-MS referred to one of the authors' previous studies (7).

Standard solutions purchased from Research Institute of Beijing North Weiye Metrology Technology[1] were used for calibration. The product numbers of these standard solutions were as follows: Se, GBW(E)080136; Cd, GBW(E)080005; Hg, GBW(E)082530; As, GBW08611; Pb, GBW08619; Cr, GBW08614; and Zn, GSB 04-1761-2004. When the deviation of the test was less than 5%, it was considered a qualified method.

pH and organic matter content determination

Sieved soil was mixed with distilled water at the soil–water ratio of 1:5 (w/v), and the pH of the turbid liquid was measured by a pH meter.

Soil organic matter (SOM) content and matrix organic matter (MOM) contents of the samples were measured using an external heating method (34).

Statistical analysis

Excel 2019 was used for data processing and standard error calculation. SPSS 22.0 was used for statistical analysis, correlation analysis, significant difference analysis, one-way

1 http://www.bzwz.com/

ANOVA, regression analysis, and principal component analysis. The contents of Se and its potential associated metals in the samples were calculated using the @risk 7.0 software, and the final results were shown as fitted values. The metal was defined as associated metal when it showed a very significant positive correlation with Se in the samples, and the correlation coefficient r-value > 0.5. The establishment and validation of the prediction model were analyzed using software, such as EViews 10.0 and SPSS 22.0. Python, Origin 2020, and Cytoscape were used for drawing.

Results

Screening of associated metals of selenium in four kinds of selenium-rich agro-foods

The Pearson correlation coefficients between Se and the six analyzed metals, including Cr, Hg, Zn, Cd, Pb, and As, in Se-rich rice, garlic, black fungus, and egg were calculated to screen which metals were associated with Se. The results are shown in **Figure 2**, and the detailed correlation coefficients (r-value) are listed in **Supplementary Table 1**.

In Se-rich rice, Se showed highly significant positive correlation with Cd (r-value = 0.779, **Figure 2A**). Se content was also significantly correlated with the contents of Cr, As, and Zn, but the correlations were weak (r-value <0.3). In Se-rich garlic, Se was correlated with Cd, Zn, and Cr, and the correlation between Se and Cd (r-value = 0.610, **Figure 2B**) was the strongest. In Se-rich black fungus, Se was significantly correlated with Cr, As, and Zn, with the strongest correlation observed between Se and Cr (r-value = 0.694, **Figure 2C**). Some significant associations were also observed between metals. For example, Cd and Hg was highly associated (r-value = 0.706), and As was associated with Cd, Cr, Hg, and Pb. In Se-rich egg, Se was significantly correlated with Cr and Zn. The correlation between Se and Cr (r-value = 0.541, **Figure 2D**) was stronger than that between Se and Zn (r-value = 0.376). Some metals were strongly correlated. For example, the r-values between Cd and Cr, Cd and Hg, Cr and Hg, Cr and As, and Pb and Zn were all higher than 0.5.

Screening of factors influencing the accumulation of selenium and its main associated metal in four kinds of selenium-rich agro-foods

Scatter plot and regression analysis were performed to reveal the main factor affecting the accumulation of Se and its main associated metal in Se-rich agro-foods.

The Se content in Se-rich rice grain was positively correlated with the Se content in soil, soil pH, and SOM, with corresponding r-values of 0.7729, 0.8133, and 0.8342, respectively (**Figures 3A–C**). The Cd content in Se-rich rice grain was positively correlated with the Cd content in the soil and SOM but negatively correlated with the soil pH, with corresponding r-values of 0.8963, 0.9501, and −0.8736, respectively (**Figures 3E–G**). The Se and Cd contents in Se-rich rice were not significantly correlated with those in irrigation water (**Figures 3D,H**).

The Se content in Se-rich garlic was positively correlated with the Se content in soil, soil pH, and SOM, with corresponding r-values of 0.8639, 0.5557, and 0.6113, respectively (**Figures 4A–C**). The Cd content in Se-rich garlic was positively correlated with the Cd content in soil and SOM but was negatively correlated with soil pH, with corresponding r-values of 0.8944, 0.7507, and −0.7006, respectively (**Figures 4E–G**). The Se and Cd contents in Se-rich garlic were not significantly correlated with those in irrigation water (**Figures 4D,H**).

The Se content in Se-rich black fungus was positively correlated with the Se content in the matrix and MOM, with corresponding r-values of 0.8993 and 0.9118, respectively (**Figures 5A,B**). The Cr content in Se-rich black fungus was positively correlated with the Cr content in the matrix and MOM, with corresponding r-values of 0.7879 and 0.9329, respectively (**Figures 5E,F**). The Se and Cr contents in Se-rich black fungus were not significantly correlated with those in irrigation water nor the pH of the matrix (**Figures 5C,D,G,H**).

The Se and Cr contents in Se-rich egg were positively correlated with those in the feed (**Figures 6A,C**), with corresponding r-values of 0.8952 and 0.7979, respectively. The Se and Cr contents in Se-rich egg were not significantly correlated with those in feeding water (**Figures 6B,D**).

Construction and validation of prediction models for the accumulation of selenium and Cd in selenium-rich rice

Based on the analysis described above, the accumulation of Se in Se-rich rice grains (Se_{rice}) was mainly associated with the total amount of Se in the soil (Se_{soil}), soil pH (pH_{soil}), and SOM. Therefore, the influence of Se_{soil}, pH_{soil}, and SOM on Se_{rice} was modeled using three-factor multiple linear regression. Two models were established as follows: model I, $Se_{rice} = \alpha + \beta_1 \cdot Se_{soil} + \beta_2 \cdot pH_{soil} + \beta_3 \cdot SOM$; model I, $\lg(Se_{rice}) = \alpha + \beta_1 \cdot \lg(Se_{soil}) + \beta_2 \cdot \lg(pH_{soil}) + \beta_3 \cdot \lg(SOM)$. The parameters of these two models are presented in **Table 2**. The R^2 and adjusted R^2 (R^2-adj) of these two prediction models were all close to 0.9, indicating that the two models had a high degree of fitting and were valid. Although the R^2 and R^2-adj

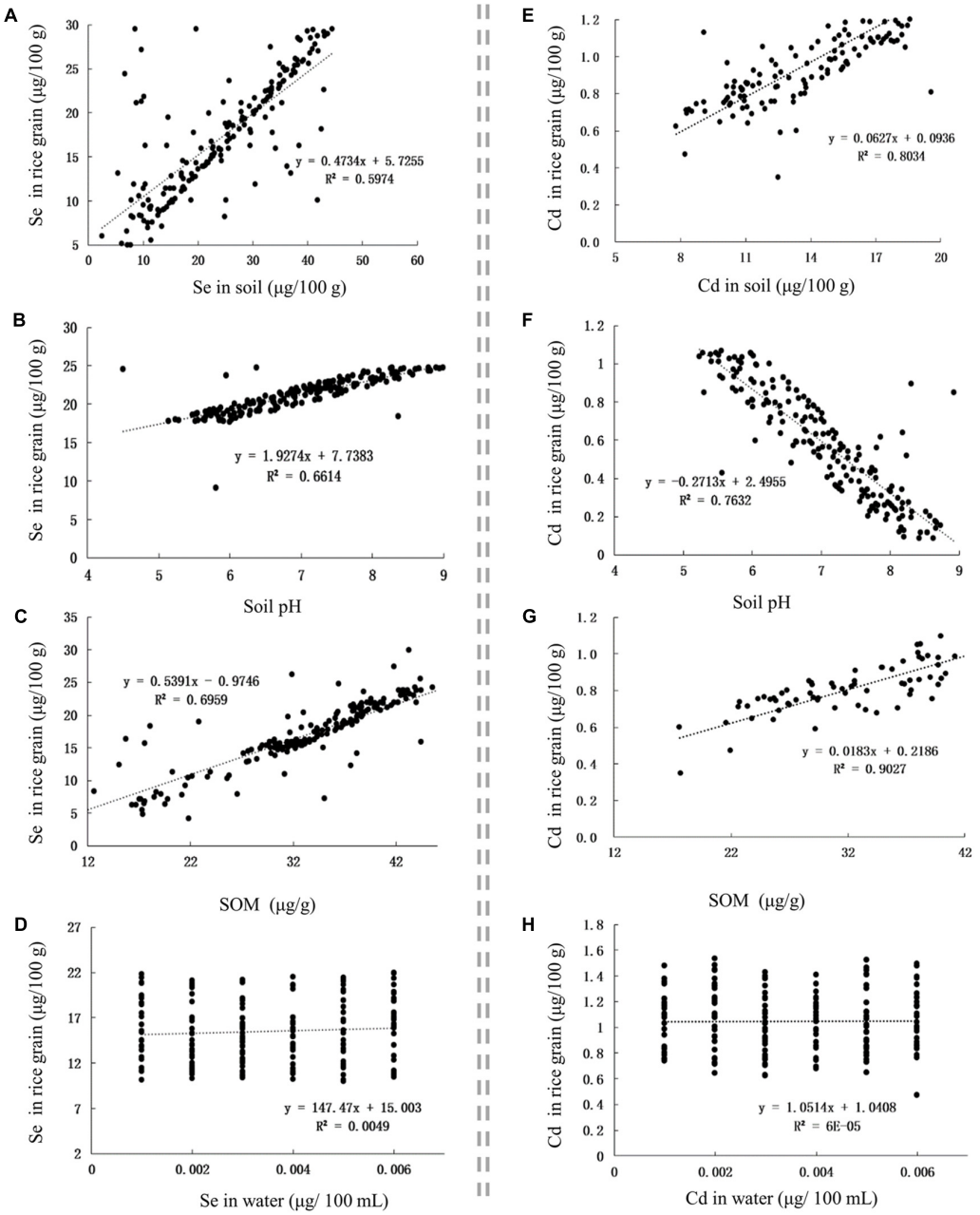

FIGURE 3
The influence of factors on Se and Cd contents in rice grain. **(A–D)** The influence of Se content in soil, soil pH, SOM, and Se content in water on Se content in rice grain, respectively. **(E–H)** The influence of Cd content in soil, soil pH, SOM, and Cd content in water on Cd content in rice grain, respectively.

in model I were slightly bigger than those in model II, the confidence of the $lg(pH_{soil})$ and constant α of model II were much higher than those of the pH_{soil} and constant α of model II. Besides, the sample variance of model II was much smaller than that of model I. Therefore, model II is more suitable to predict the Se content in Se-rich rice, and the equation was as follows: $lg(Se_{rice}) = -0.1983 + 0.1022\ lg(Se_{soil}) + 0.1697\ lg(pH_{soil}) + 0.7545\ lg(SOM)$.

The accumulation of Cd in Se-rich rice grains (Cd_{rice}) was mainly associated with the total amount of Cd in the soil (Cd_{soil}), pH_{soil}, and SOM. Therefore, the influence of Cd_{soil}, pH_{soil}, and SOM for Cd_{rice} was also modeled using three-factor multiple linear regression. Two models were established as follows: model I, $Cd_{rice} = \alpha + \beta_1 \cdot Cd_{soil} + \beta_2 \cdot pH_{soil} + \beta_3 \cdot SOM$; model II, $lg(Cd_{rice}) = \alpha + \beta_1 \cdot lg(Cd_{soil}) + \beta_2 \cdot lg(pH_{soil}) + \beta_3 \cdot lg(SOM)$. The parameters of these two models are also presented in Table 2.

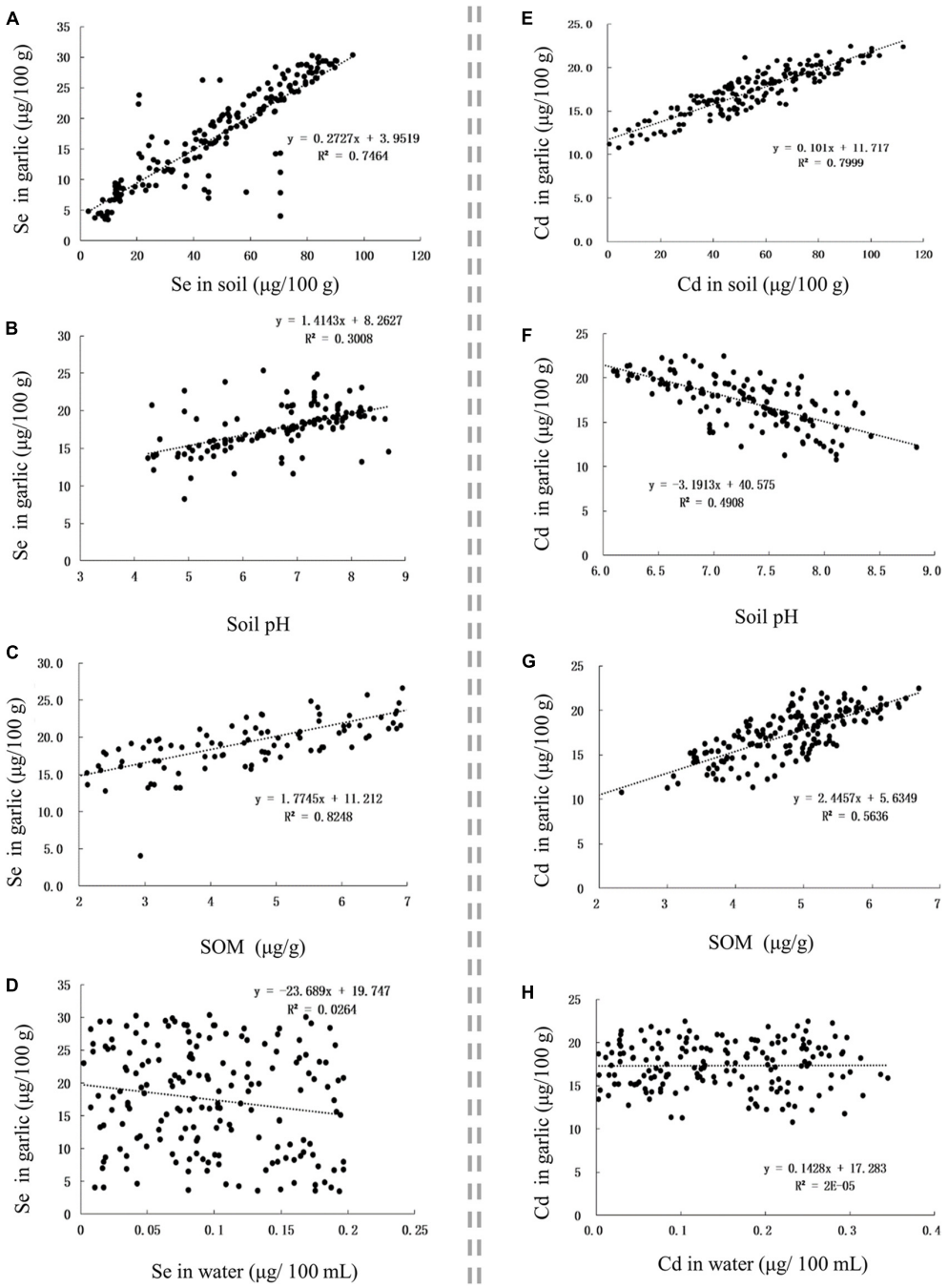

FIGURE 4
The influence of factors on Se and Cd contents in garlic. **(A–D)** The influence of Se content in soil, soil pH, SOM, and Se content in water on Se content in garlic, respectively. **(E–H)** The influence of Cd content in soil, soil pH, SOM, and Cd content in water on Cd content in garlic, respectively.

The R^2 and R^2-adj of these two prediction models were close to 0.6. The confidence of pH_{soil} of model I was 0.3 > 0.05, while the Cd content in Se-rich rice was significantly and negatively correlated with pH_{soil} (Figure 3F). Therefore, model II is more suitable to predict the Cd content in Se-rich rice, and the equation was as follows: $\lg(Cd_{rice}) = -0.1748 + 0.8605 \lg(Cd_{soil}) - 0.1695 \lg(pH_{soil}) + 0.3581 \lg(SOM)$.

The content of Se and its associated metals in blind samples were analyzed to verify the accuracy of the constructed model for predicting Se and its main associated metals in Se-rich

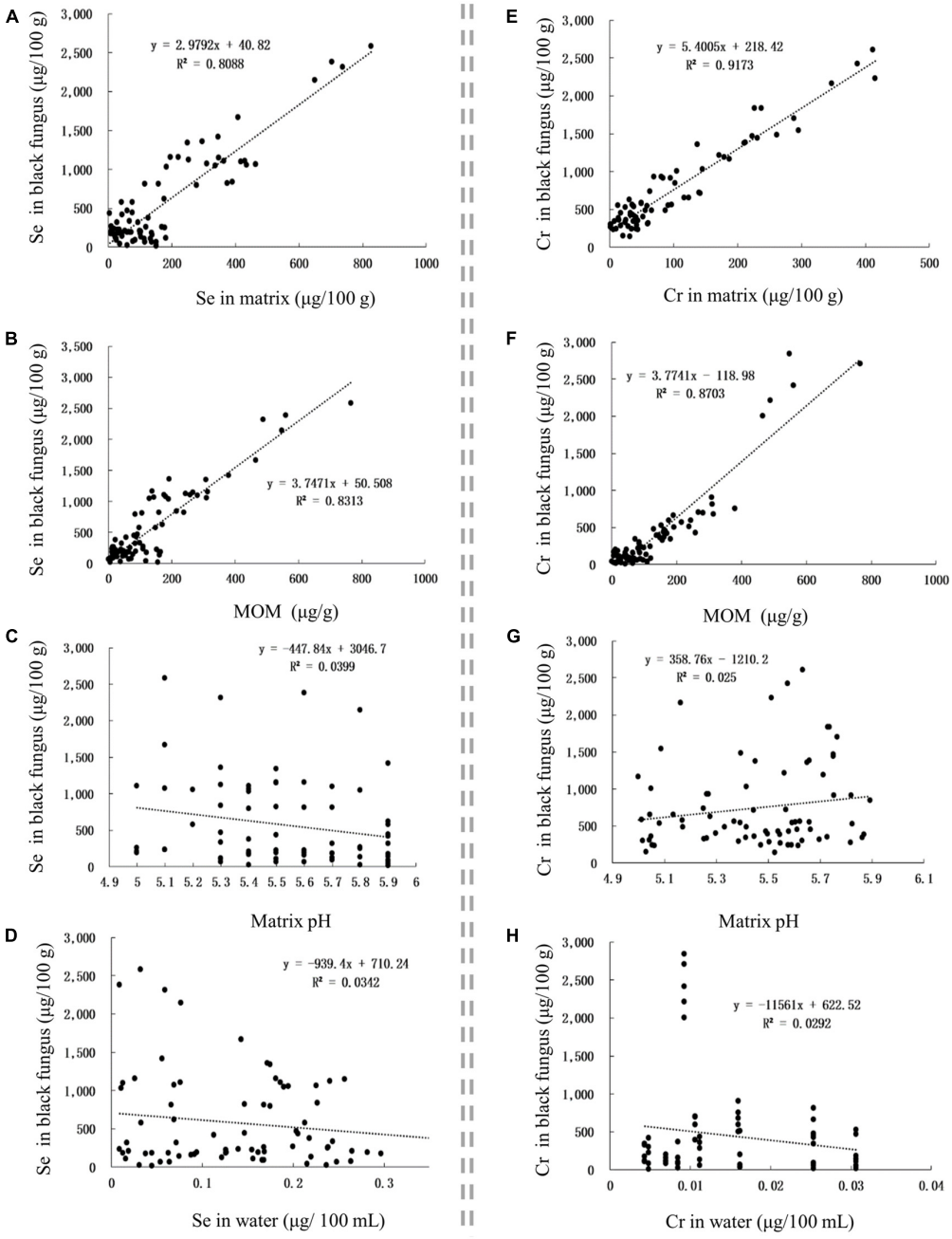

FIGURE 5
The influence of factors on Se and Cr contents in black fungus. **(A–D)** The influence of Se content in matrix, MOM, matrix pH, and Se content in water on Se content in black fungus, respectively. **(E–H)**, The influence of Cr content in matrix, MOM, matrix pH, and Cr content in water on Cr content in black fungus, respectively.

agro-foods, and then correlation and residual analyses were performed on the measured and predicted values.

The correlation between the predicted and measured values of Se and Cd in Se-rich rice was extremely significant ($p < 0.01$), and the corresponding r-values were 0.9215 and 0.7815, respectively (**Figures 7A,B**). This result indicated that the content of Se and Cd in the soil, soil pH, and SOM could well predict the accumulation of Se and Cd in Se-rich rice. The linear regression equations between the measured values (Se_m and Cd_m) and predicted values (Se_p and Cd_p) were as follows: $Se_m = 0.8736\ Se_p + 2.0074$ and $Cd_m = 0.6765\ Cd_p + 0.2999$. The residual distribution of the models was analyzed to further investigate their rationality. The residuals of Se and Cd contents were evenly distributed on both sides of the $Y = 0$ axis and

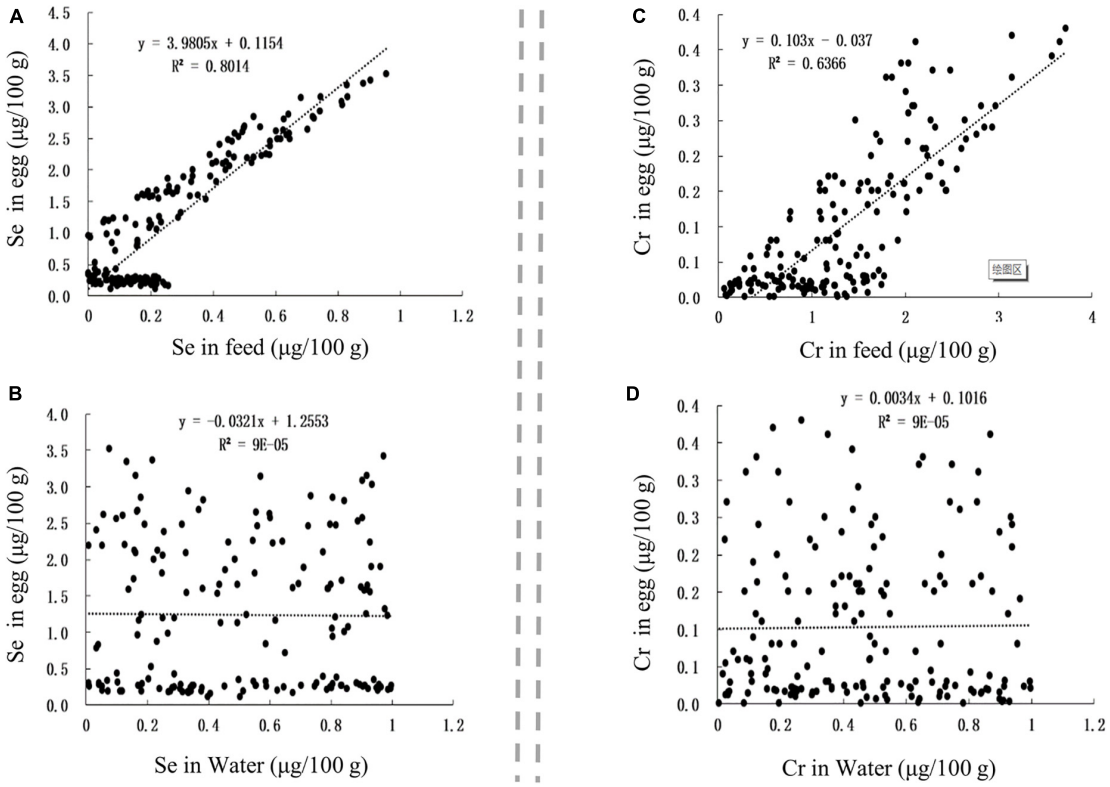

FIGURE 6
The influence of factors on Se and Cr contents in egg. **(A,B)** The influence of Se content in feed and Se content in water on Se content in egg, respectively. **(C,D)** The influence of Cr content in feed and Cr content in water on Cr content in egg, respectively.

TABLE 2 Prediction models of Se and Cd accumulation in Se-rich rice grain.

Model	Variate	Coefficient	R^2	R^2_adj	T statistic	*P*	Sample variance
Model I Se_{rice}	Se_{soil}	0.0958	0.8926	0.8944	2.7639	0	3.7724
	pH_{soil}	0.2204			2.1448	0.0333	
	SOM	0.0404			17.5977	0.0012	
	C	−0.07635			−0.1307	0.8962	
Model II Se_{rice}	$lg(Se_{soil})$	0.1022	0.8880	0.8898	4.7548	0	0.1037
	$lg(pH_{soil})$	0.1697			3.5706	0.0005	
	lg(SOM)	0.7545			16.5594	0.0001	
	C	−0.1983			−4.4942	0	
Model I Cd_{rice}	Cd_{soil}	0.0198	0.5857	0.5787	10.8973	0.0003	0.0542
	pH_{soil}	−0.0033			−1.0141	0.3119	
	SOM	0.0008			5.6177	0.0002	
	C	0.0160			3.2413	0.0001	
Model II Cd_{rice}	$lg(Cd_{soil})$	0.8605	0.5650	0.5577	10.6695	0.0001	0.0463
	$lg(pH_{soil})$	−0.1695			−2.5123	0.0129	
	lg(SOM)	0.3581			5.1465	0.0002	
	C	−0.1748			−1.5140	0.1318	

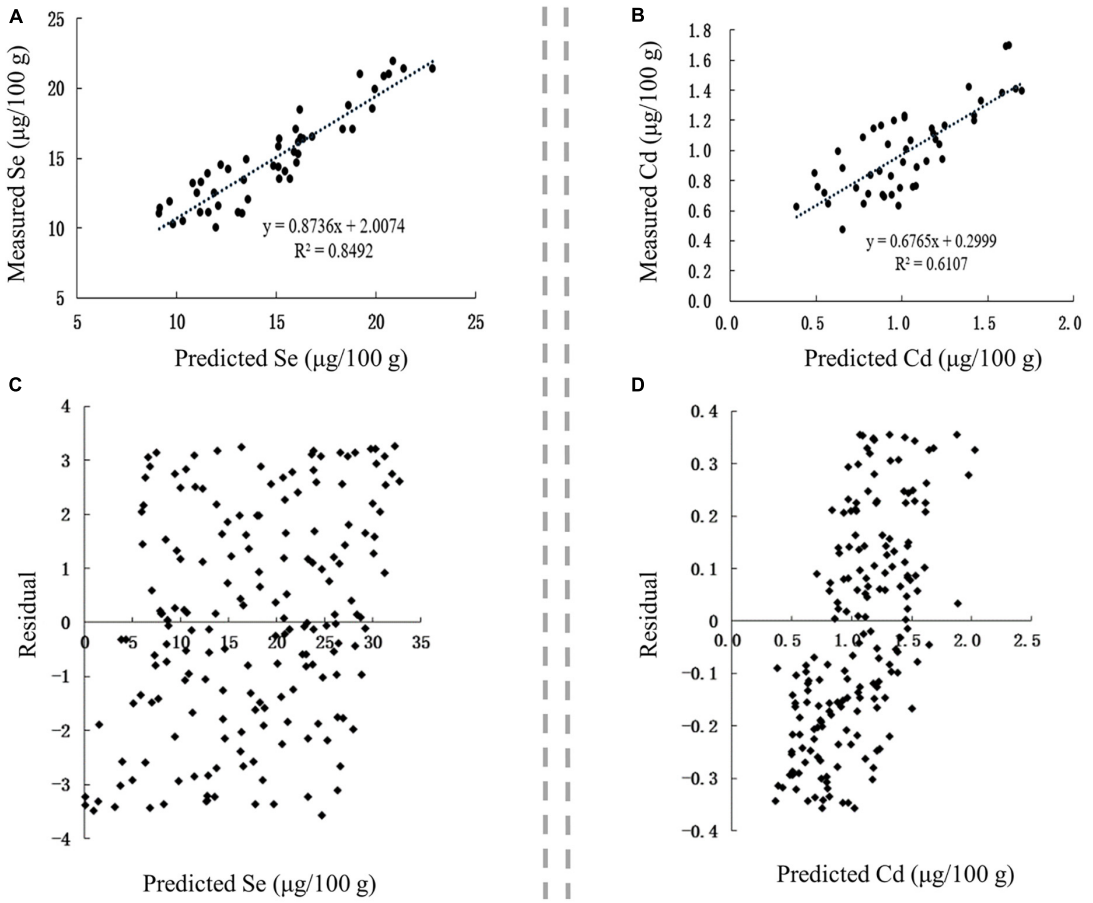

FIGURE 7
Validation of prediction models in Se-rich rice grain. **(A,B)** Correlation between the fitted and measured Se and Cd contents, respectively. **(C,D)** Residual analysis of the fitted and measured Se and Cd contents, respectively.

concentrated in ± 4 and ± 0.4, respectively (Figures 7C,D). Collectively, the established models for predicting Se and Cd contents in Se-rich rice were reasonable and reliable.

Construction and validation of prediction models for the accumulation of selenium and Cd in selenium-rich garlic

The influence of Se_{soil}, pH_{soil}, and SOM for Se_{garlic} and that of Cd_{soil}, pH_{soil}, and SOM for Cd_{garlic} were modeled using three-factor multiple linear regression. The processes of modeling and model screening were the same as those for Se_{rice} and Cd_{rice} as described above. The parameters of these models are presented in Table 3.

The R^2 and R^2-adj of the two prediction models for Se_{garlic} were all close to 1, indicating that these two models had a high degree of fitting and were valid. The confidences of variances of models I and II were close, while the coefficient of SOM in model I was much higher than that of lg(SOM) in model II. Therefore, model I is more suitable to predict the Se content in Se-rich garlic, and the equation was as follows: $Se_{garlic} = 5.0587 + 0.2649\ Se_{soil} + 0.3258\ pH_{soil} + 0.2119$ SOM.

The R^2 and R^2-adj of model I for Cd_{garlic} were close to 1, much higher than those of model II, indicating that model I had a higher degree of fitting than model II. Besides, the confidences of variances of model I were higher than those of model II, and the confidence of lg(SOM) in model II was $0.079 > 0.05$. Thus, model I is more suitable to predict the Cd content in Se-rich garlic, and the equation was as follows: $Cd_{garlic} = 15.0199 + 0.0933\ Cd_{soil} - 0.4722\ pH_{soil} + 0.3434$ SOM.

The correlation between the predicted and measured values of Se and Cd in Se-rich garlic was extremely significant ($p < 0.01$), with corresponding r-values of 0.9565 and 0.7843, respectively (Figures 8A,B). The linear regression equations between the measured values (Se_m and Cd_m) and predicted values (Se_p and Cd_p) were as follows: $Se_m = 0.9215\ Se_p + 1.8114$ and $Cd_m = 0.5447\ Cd_p + 8.0479$. The residuals of Se and Cd

contents were evenly distributed on both sides of the $Y = 0$ axis and concentrated in ± 4 (**Figures 8C,D**). Collectively, the established models for predicting Se and Cd contents in Se-rich garlic were reasonable and reliable.

Obviously, the prediction models for Se and Cd in rice and garlic showed the relationship with the metal contents, pH, and SOM in soil, which could be attributed to the soil characteristics in seleniferous areas.

Construction and validation of prediction models for the accumulation of selenium and Cr in selenium-rich black fungus and eggs

The accumulation of Se and Cr in Se-rich black fungus was mainly associated with the total amount of Se and Cr in the matrix and MOM. The prediction models of Se_{fungus} and Cr_{fungus} were established using two-factor multiple linear regression. The parameters of these models are presented in **Table 4**.

The R^2 and R^2-adj of model I for Se_{fungus} were close to 0.9, much higher than those of model II, indicating that model I had a higher degree of fitting than model II. Therefore, model I is more suitable to predict the Se content in Se-rich black fungus, and the equation was as follows: $Se_{fungus} = -1.4197 + 1.5109\ Se_{matrix} + 2.1829$ MOM.

The R^2 and R^2-adj of model I for Cr_{fungus} were close to 1, much higher than those of model II, indicating that model I had a higher degree of fitting than model II. Therefore, model I is more suitable to predict the Cr content in Se-rich black fungus, and the equation was as follows: $Cr_{fungus} = -12.1461 + 3.9994\ Cr_{matrix} + 0.7777$ MOM.

The correlation between the predicted and measured values of Se and Cr in Se-rich black fungus was extremely significant ($p < 0.01$), with corresponding r-values of 0.9565 and 0.9463, respectively (**Figures 9A,B**). The linear regression equations between the measured values (Se_m and Cr_m) and predicted values (Se_p and Cr_p) were as follows: $Se_m = 0.9783\ Se_p59.388$ and $Cr_m = 0.9515\ Cr_p + 37.612$. The residuals of Se and Cr contents were concentrated in ± 500 and ± 300, respectively (**Figures 9C,D**). Collectively, the established models for predicting Se and Cr content in Se-rich black fungus were reasonable and reliable.

Due to limited variables, the prediction model could be generated as linear regression equation for Se content in Se-rich egg (Se_{egg}) on the basis of Se content in the feed (Se_{feed}) as follows: $Se_{egg} = 0.1154 + 3.9805\ Se_{feed}$, and the R^2 was 0.8014. The linear regression equation for Cr content in Se-rich egg (Cr_{egg}) based on Se content in the feed (Cr_{feed}) was $Cr_{egg} = -0.037 + 0.103\ Cr_{feed}$, and the R^2 was 0.6366.

The correlation between the predicted and measured values of Se and Cr in Se-rich egg was extremely significant ($p < 0.01$), with corresponding r-values of 0.8511 and 0.8590, respectively (**Figures 10A,B**). The linear regression equations between the measured values (Se_m and Cr_m) and predicted values (Se_p and Cr_p) were $Se_m = 0.6125\ Se_p + 0.9328$ and $Cr_m = 0.8494\ Cr_p + 0.0882$. The residuals of Se and Cr contents were concentrated in ± 1 and ± 0.2, respectively (**Figures 10C,D**). Collectively, the established models for predicting Se and Cr content in Se-rich egg were reasonable and reliable.

Different from rice and garlic samples, the Se and Cr distribution in black fungus and eggs was mainly influenced by the culture medium and feed instead of soil conditions.

Discussion

Metals association phenomenon often occurs in seleniferous areas, leading to the associated metals pollution in Se-rich agro-food, and types of related heavy metals differ in various agro-foods (14). Therefore, the association analysis of Se and heavy metals in various agro-foods has important values. In this study, representative main Se-rich producing areas in China were selected to investigate and collect Se-rich rice, eggs, black fungus, and garlic samples and Se-rich soil, matrix, feed, irrigation, and feeding water samples. The typical associated metals with Se in these agro-foods have been screened, the decisive factors for their accumulation have been determined, and the corresponding prediction models also have been constructed.

Cd association in selenium-rich rice grain and garlic

The most correlated heavy metal has been confirmed to be Cd in both rice grain and garlic in our results. In many natural seleniferous areas, soils are contaminated by heavy metals caused by the weathering of Se-rich shales (35, 36). In particular, Cd has the highest bioavailability, with bioavailable fractions that could reach up to 41.84% of the total Cd in soils; the bioaccumulation factors (BAFs) for crops follow the order Cd > Zn > As > Cu > Ni > Hg > Cr > Pb, making the development of seleniferous area risky (35, 37, 38). In many plants, Se is always applied as an inhibitor for Cd biological toxicity, and Se at specific concentration and valence could efficiently mitigate the oxidative stress induced by Cd, making them coexist in rice (20, 39). The correlation between Se and Cd in rice grain has also been confirmed in the previous work by pot experiment and transcriptome analysis (23). Garlic, which could metabolize inorganic Se into Se amino acids, is an important source for Se amino acid absorption (40, 41). As a plant with good resistance to biotic and abiotic environmental stresses, garlic showed strong resistance to Cd stress, which could be the reason for the Cd accumulation (42).

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TABLE 3 Prediction models of Se and Cd accumulation in Se-rich garlic.

Model	Variate	Coefficient	R^2	R^2_adj	T statistic	*P*	Sample variance
Model I Se_{garlic}	Se_{soil}	0.2649	0.9682	0.9677	72.6167	0	6.7748
	pH_{soil}	0.3258			1.9247	0.0559	
	SOM	0.2119			1.9271	0.0556	
	C	5.0587			3.2467	0.0014	
Model II Se_{garlic}	$lg(Se_{soil})$	0.4615	0.9438	0.9428	53.8424	0.0001	0.3425
	$lg(pH_{soil})$	0.1421			1.8802	0.0617	
	lg(SOM)	0.0590			3.3324	0.0010	
	C	0.9348			5.6488	0	
Model I Cd_{garlic}	Cd_{soil}	0.0933	0.9889	0.9890	101.5714	0.0003	2.1797
	pH_{soil}	−0.4722			−14.2108	0.0001	
	SOM	0.3434			14.9616	0.0002	
	C	15.0199			50.2466	0.0001	
Model II Cd_{garlic}	$lg(Cd_{soil})$	0.1726	0.7385	0.7340	16.64448	0.0001	0.1486
	$lg(pH_{soil})$	−0.4420			−5.9368	0.0002	
	lg(SOM)	0.0319			1.7658	0.0792	
	C	2.4357			14.5825	0.001	

The accumulation situation of Se and Cd in rice grain and garlic also showed similarity. Their Se content was positively correlated with the Se content in soil, soil pH, and SOM, and their Cd content was positively correlated with Cd content in soil and SOM, and negatively correlated with soil pH. These results were consistent with those of previous studies, that is, the higher the content of Se and Cd in soil, the higher the content of Se and Cd in crops (43). With the increase in soil pH, the transformation of soluble selenate to partially soluble selenite has been inhibited, which further increased the formation of water-soluble Se (44). Meanwhile, the content of water-soluble Cd in soil decreased (45), which may explain why the Se and Cd contents in rice were positively and negatively correlated with soil pH, respectively. Besides, SOM can fix and increase the Se and Cd contents in soil, further enhancing the bioavailability of Se and Cd (46–48).

As reported, the Se content in soil has a high correlation with SOM and pH, and soil Se and pH are the most important parameters that influence Se uptake of crops and vegetables (49, 50). In this study, the Se content in rice grain and garlic was positive influenced by soil pH and SOM, which could also explain the previous results that the application of fertilizer could efficiently increase the Se content in plants (51, 52). Stroud et al. (53) constructed a regression model of Se content in wheat grain, and the total soil Se, extractable soil Se, and extractable soil S were suggested as main controlling parameters for grain Se (Grain Se concentration = −10.32 + 0.1085 × (total soil Se)−1.9 × (extractable soil Se) + 2.515 × (extractable soil S)). The spatial distribution of Se in grain was consistent with that of bioavailable soil Se, which was found to be dominantly influenced by soil pH and SOM (54). However, Gu et al. (37) constructed models for the prediction of BAFs of Se in rice grain and found that BAFs of Se were negatively correlated with soil pH and TOC, which could be related to the difference among sampling sites.

There are more predictive models that have been established for Cd accumulation in rice, focusing on Cd bioavailability influenced by a diversity of soil properties. Soil pH could be the most important factor affecting Cd content in rice grain. In this study, soil pH was negatively correlated with the Cd content in rice grain and garlic. Similarly, Yang et al. (55) reported a Cd prediction model for rice in tin mining area, and the Cd concentration in rice was significantly negative related to the soil pH, and also influenced by CaO, TOC, and Mn in soil. In rice grown both in karst and non-karst areas, the Cd concentration in rice grain was also negative related to the soil pH, and affected by CaO, and Mn in soil (56). The prediction model built by Tang et al. (57), also suggested the Cd content was negatively influenced by soil pH and TFe_2O_3 content. The models for Cd prediction in crops exhibit some commonality on the factors. A prediction model of wheat grain Cd was constructed with the variables of soil Cd, soil pH, soil Ca, and coexisting Zn (58). Prediction models of maize and peanut Cd were also determined by TOC, pH, and Mn

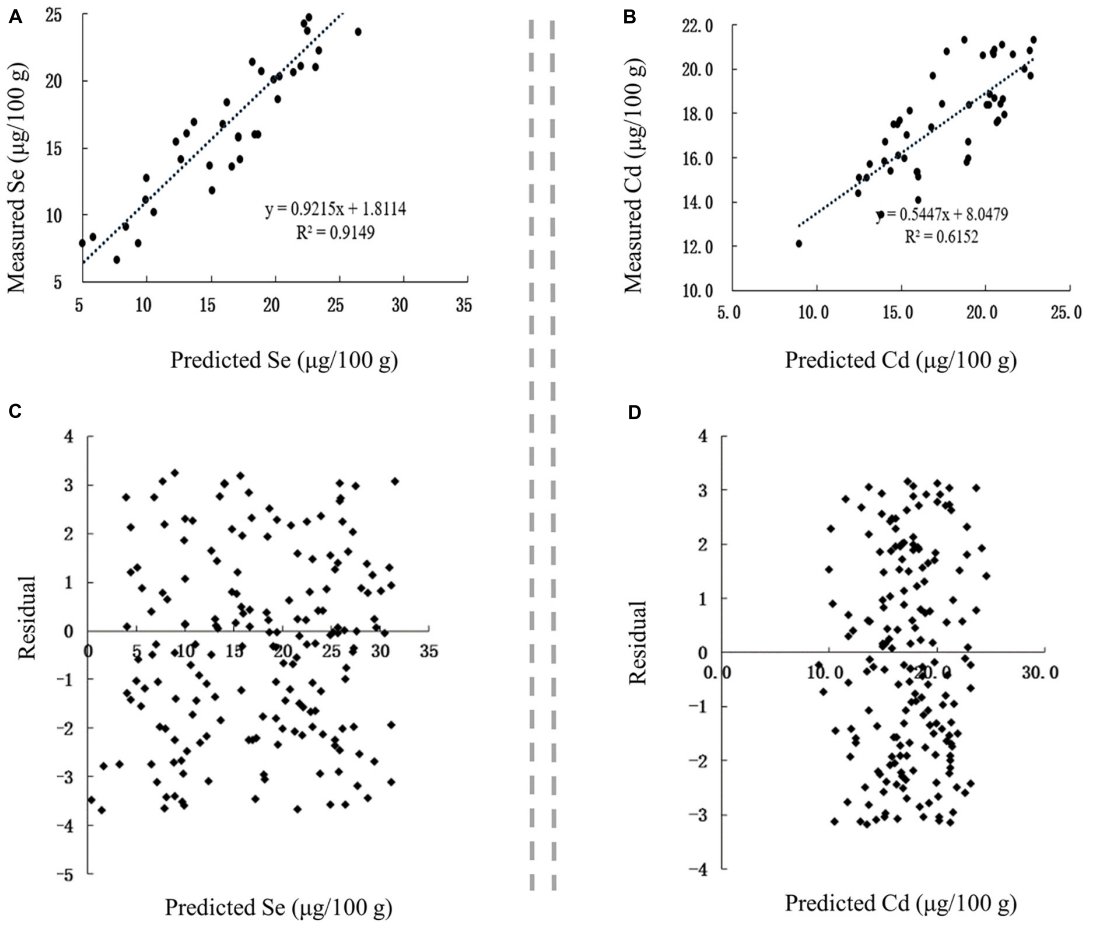

FIGURE 8
Validation of prediction models in Se-rich garlic. **(A,B)** Correlation between the predicted and measured Se and Cd contents, respectively. **(C,D)** Residual analysis of the fitted and measured Se and Cd contents, respectively.

content in soil (59). Soil Cd content, TOC, and pH values significantly affected the Cd bioaccumulation in sugarcane (57). Interestingly, all of these models exhibited negative relations between Cd accumulation and soil pH, which means the artificial increase of soil pH could be an efficient way to enhance the Se content and decrease the Cd accumulation in the crops at the same time.

The accumulation of many other metals in crops also correlated with the soil properties, such as pH, TOC, cation exchange capacity (CEC), CaO content and Mn content. The predication models showed that Pb content in wheat grain and pepper, and Zn content in rice grain were all negatively influenced by soil pH (33, 60, 61). The BAFs of Cu, Pb, Zn in maize and peanut were negative related with the Mn content in soil (59). Moreover, to improve the application potential, more data covering various varieties, regions and seasons need to be obtained for the construction of prediction models.

Cr association in selenium-rich black fungus and egg

Edible fungi are good sources of dietary Se, and their Se content could be effectively improved using Se-enriched fermentation culture medium (62). Exogenous Se in organic (Se-enriched yeast) and inorganic (selenite and selenate) forms could be utilized by fungi (63). The addition of dietary Se yeast and selenite supplementation could also increase Se content in laying Longyan duck eggs and Nile tilapia fish tissues (64, 65).

However, edible fungi are detected to have higher heavy metal content compared to crops (66). Therefore, the standards for heavy metal contents in edible fungi were higher than that of crops and egg (**Table 1**). Mediums containing wheat straw and rice bran are common substrates for edible fungi grown, and they accumulate more heavy metals than corresponding grains. Meanwhile, the raw material pollution, the machinery and equipment exposure during manufacture and environment make the mineral pollution in medium and animal feeds is

TABLE 4 Prediction models of Se and Cr accumulation in Se-rich black fungus.

Model	Variate	Coefficient	R^2	R^2_adj	T-statistic	*P*	Sample variance
Model I Se_{fungus}	Se_{matrix}	1.5109	0.8944	0.8914	6.5631	0.0001	602.1944
	MOM	2.1829			7.6430	0.0002	
	C	−1.4197			−0.04337	0.9655	
Model II Se_{fungus}	$lg(Se_{matrix})$	0.2908	0.5599	0.5477	3.3059	0.0015	0.5131
	lg(MOM)	0.5672			6.7161	0.0002	
	C	0.8411			4.5240	0.0001	
Model I Cr_{fungus}	Cr_{matrix}	3.9994	0.9653	0.9643	11.4330	0.0001	388.0355
	MOM	0.7777			4.7787	0.0002	
	C	−12.1461			−1.0336	0.3048	
Model II Cr_{fungus}	$lg(Cr_{matrix})$	0.6051	0.7148	0.7069	7.5480	0.0001	0.5419
	lg(MOM)	0.3175			3.7705	0.0003	
	C	0.7399			5.7031	0.0002	

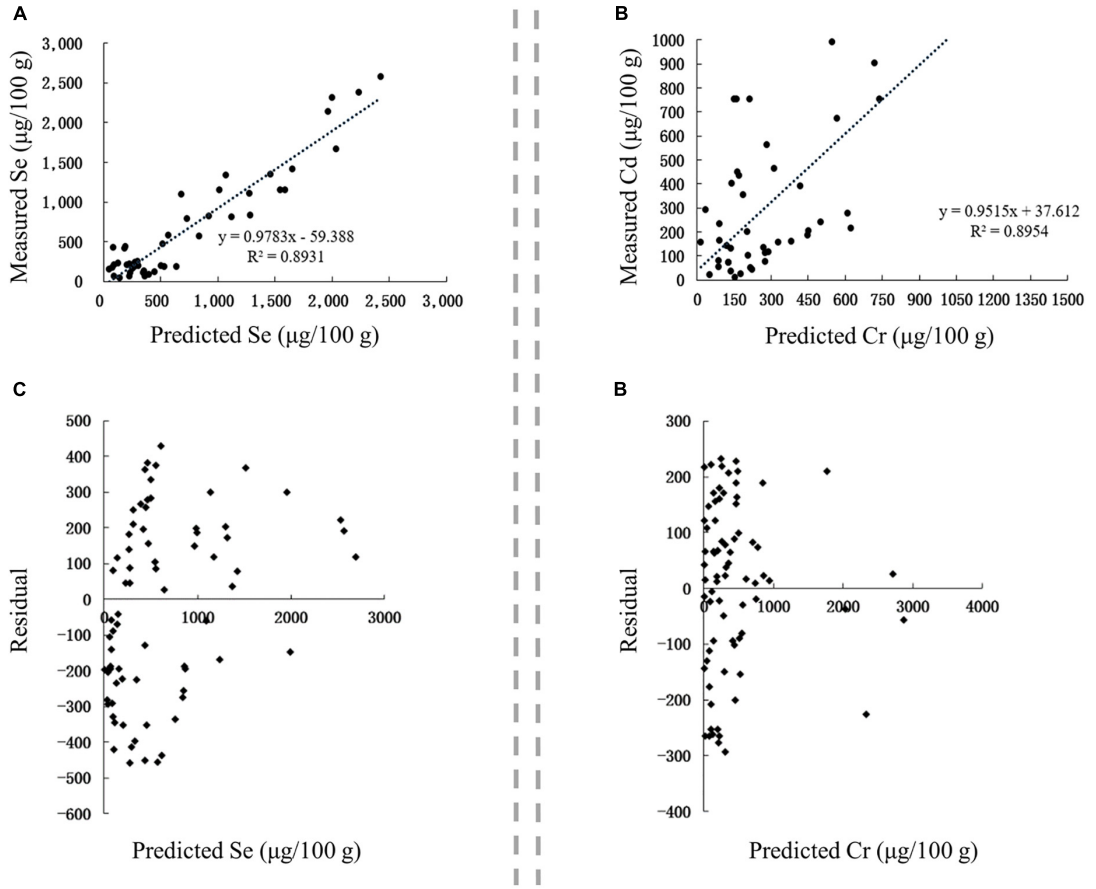

FIGURE 9
Validation of prediction models in Se-rich black fungus. **(A,B)** Correlation between the predicted and measured Se and Cr contents, respectively. **(C,D)** Residual analysis of the fitted and measured Se and Cr contents, respectively.

unavoidable (67). In this study, Cr has been proved as the most associated heavy metal in Se-rich black fungus and egg. Hu et al. (68) reported that Se could significantly reduce the accumulation of Cr in fruiting bodies of black fungus, which may be related to the coexistence of Cr and Se. The health risk assessment of heavy-metal contamination in Se-rich eggs also indicated that Cr was the most correlated heavy metal in Se-rich eggs (69).

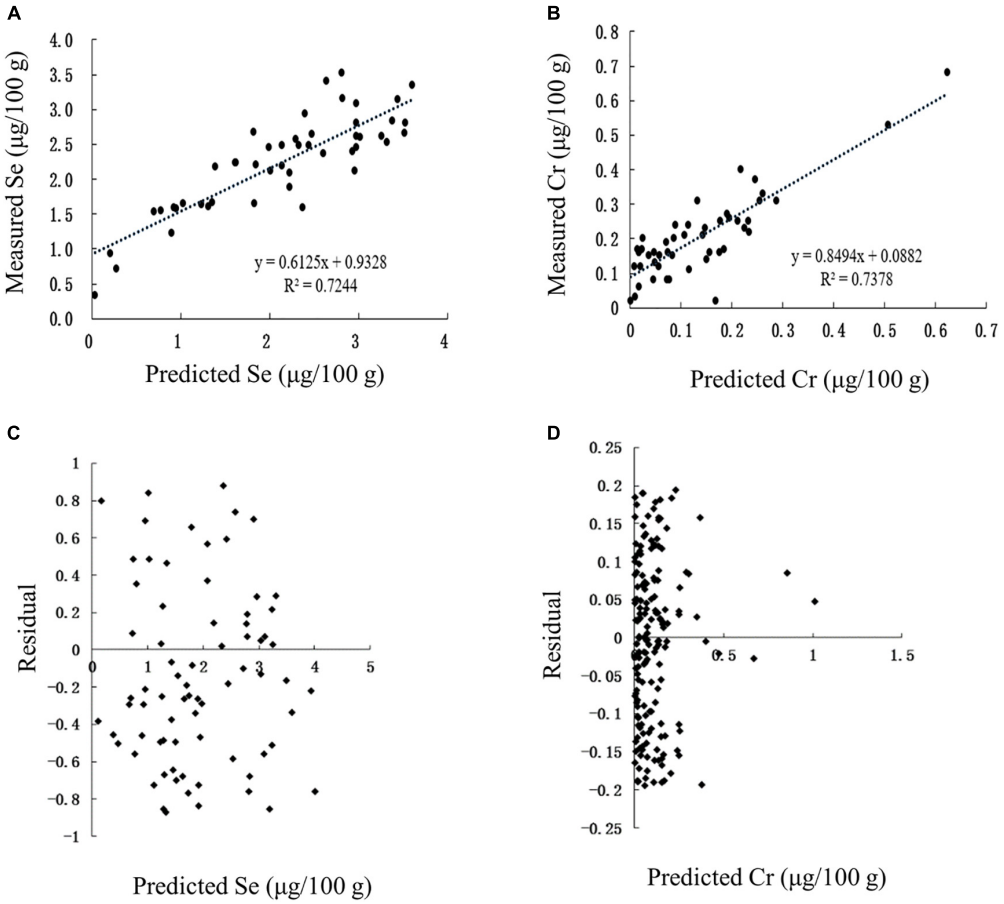

FIGURE 10
Validation of prediction models in Se-rich egg. **(A,B)** Correlation between the predicted and measured Se and Cr contents, respectively. **(C,D)** Residual analysis of the fitted and measured Se and Cr contents, respectively.

Our results showed that Se and Cr content in black fungus were positively correlated with the Se and Cr content in the matrix and MOM. Fungi are sensitive to Se and metals in medium; thus, they could be used to indirectly reflect the pollutants in media, such as water, atmosphere, and soil (70–72). With the addition of increasing levels of selenite, the total Se content in golden needle mushroom (*Flammulina velutipes*) fruiting body also increased (73). The metal intake by fungi is also related to the pH and organic matter contents in the medium (74). Se and Cr accumulation in egg were mainly affected by the Se and Cr content in feed. These results agreed with those of the previous studies, that is, dietary Se supplementation could increase the total egg Se levels, and the total egg Se levels increased with the improvement of the dietary Se supplementation (75, 76). Heavy-metal accumulation in eggs had a positive correlation with the intake of feed contaminated with heavy metals (77), similar to that in fishes and other animals (78). Moreover, a previous study has found that Cr could aggregate more easily than other heavy metals in eggs. When under the same feeding conditions, the Cr content in eggs was higher than that in meat products and liver, which presented higher risks (67). The prediction models of Cr in black fungus and egg could be used for the safety assessment of cultural medium and feeds for Se-rich fungus and eggs. In summary, the common ground of heavy-metal accumulation in rice grain and garlic indicate that the detection and treatment of Cd pollution are essential before the development of Se-rich land resources; and the Cr pollution in mushroom medium and poultry feed should not also be ignored. The prediction model of heavy metals in the agro-foods could be used for the assessment of Se-rich soils, cultural medium and feeds.

Conclusion

Some metals were associated with Se in Se-rich agro-foods. The main associated metal in Se-rich rice grain and garlic was Cd, and the main associated metal in Se-rich black fungus and egg was Cr. The contents of Se and its associated metal in Se-rich agro-foods was highly and positively correlated with those

of the corresponding element in soil, matrix, feed, SOM, and MOM. The Se content in Se-rich rice grain and garlic was also positively correlated with soil pH, whereas the content of the main associated metals in these two agro-foods was negatively correlated with soil pH. The contents of Se and metals in irrigation waters for plants and feeding water for hens had no significant effect on the content of Se and metals in Se-rich agro-foods. Eight models for prediction of Se and its main associated metals in Se-rich rice grain, garlic, black fungus, and egg were established using multiple linear regression analysis. The R^2 and R^2-adj of each model were high. The high accuracy of these models was validated by correlation analysis between the measured values and the predicted values of the blind samples and residual analysis. In this study, the main associated metals and the main factors affecting the accumulation of Se and the main associated metals were revealed. The prediction models in four typical Se-rich agro-foods were also established and validated, providing valuable guidance to produce high-quality and healthy Se-rich agro-foods.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

LJ: conceptualization, data curation, investigation, and writing—original draft. LZ: conceptualization, resources, methodology, and data curation. YZ: investigation, software, data curation, and formal analysis. RW and XL: supervision and funding acquisition. BL: supervision, project administration, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.990628/full#supplementary-material

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Salsola soda as selenium biofortification crop under high saline and boron growing conditions

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In California, there is a shortage of good quality water available for irrigated agriculture due to severe drought. Consequently, saline groundwaters and drainage waters containing natural-occurring selenium (Se) and boron (B) salts are being considered as alternative sources of water for irrigation on salt and B tolerant crops like the edible halophyte-agretti (Salsola soda L.). In this multi-year field study, we evaluated agretti grown as a Se-biofortification crop in typical saline/B-laden soils (10 dS m^{-1} and 12 mg B/L) and irrigated with saline (3–8 dS m^{-1}) and low-saline water (<1 d/S m) containing B (3–6 mg B/L) and Se (0.02–0.25 mg Se/L) at different evaporation transpiration (Et_o) rates (100, 75, and 50 %, respectively). During the four-year study, fresh biomass yields ranged from 1 to 3 kg/m^2 and were generally highest with irrigation at 100 % Et_o with either saline or low-saline water. Tissue Se concentrations ranged from 2 to 3.2 mg Se / kg DW and 0.4–0.5 mg Se/kg DW with saline and low-saline irrigation, respectively. Selenium speciation in plant tissue showed the following: selenomethionine (SeMet) > selenate (SeO_4) > methylselenocysteine ($MeSeCy_s$), irrespective of any treatment (i.e., year of planting, saline or low saline irrigation, rate of water application, direct seeding or transplanted). Agretti did not exhibit any toxicity symptoms as indicated by changes in total phenolic concentrations. Total phenolics ranged from 180 to 257 GAE/L and showed no significant differences among all treatments, although they were generally higher at the lowest water treatment (50% Et_o). In regard to toxic ion accumulation, agretti tolerated excessive sodium (Na) and boron (B) and tissue concentrations ranging from 5.5 to 8.8% Na and 60 to 235 mg B/kg DW, respectively. Results from this multi-year study have identified a unique Se-biofortification strategy for producing Se-enriched

agretti using saline, B- and Se-laden soil and irrigating with saline and low-saline water, respectively. Successful production of this crop may promote Se- biofortification strategies in poor quality regions where natural- occurring Se is present in soils and in waters used for irrigation.

KEYWORDS

biofortification, *Salsola soda*, selenium, boron, salinity

Introduction

Presently, about 3.6 billion people suffer water scarcity each year (Boretti and Rosa, 2019) and the situation is expected to become worse in the next few decades (World Water Assessment Programme (Nations Unies), 2018), especially affecting food production. This problem is even more serious for irrigated agriculture in one of the most productive regions of the USA, the San Joaquin Valley (SJV) in Central California. In Central California, there is a shortage of good quality water available for irrigated agriculture due to severe drought, reductions in water allotments, and growing municipal, urban, and environmental demands. Hence, the identification of other water sources, which include high saline water, is considered as an alternative source to water shortages in arid regions of Central California. The potential to use high saline water for irrigation has been studied on field sites in the westside of the San Joaquin Valley (SJV) in Central California since the 1980's (Oster and Grattan, 2002; Suyama et al., 2007; Ayars and Soppe, 2014). This part of California is unique because of the existence of natural-occurring selenium (Se) and boron (B) salts in soils of the SJV and consequently in ground water and in drainage waters produced from irrigated agriculture. Their presence complicates water reuse strategies and requires selecting crops that can tolerate both the high levels of salinity and B (Díaz et al., 2013). Importantly, the additional presence of soil Se can be a double-edge sword because Se can be toxic to the biological ecosystem at excessive concentrations (as high as 1000 μg Se/L in surface water, Fordyce, 2013), although it is an essential benefit for human and animal nutrition. Selenium, as an intrinsic component of essential seleno proteins, is required in trace amounts for preserving the optimal health and balanced metabolism of mammals (Rayman, 2020). In humans, selenoproteins have functional roles in antioxidant process, protein stability, transcription of mRNA immune system, and other biochemical functions (Broadley et al., 2006; Labunskyy et al., 2014; Kieliszek, 2019). Hence, it is important for humans to maintain adequate levels of Se in their daily diet.

Consumption of meat and food crops are major sources of Se for the world's population. If natural-occurring soil Se is absorbed by a food crop, a process called Se biofortification occurs (Bañuelos et al., 2017; El-Ramady et al., 2020). Selenium biofortification can be a strategy to combat the low Se status in many parts of the world by producing food crops that are enriched with Se (Broadley et al., 2006). Generally, Se biofortification occurs when some form of Se is exogenously applied to plant or soil (Jiang et al., 2017; Smoleń et al., 2019; Luo et al., 2021), or when crops are grown in Se-laden soils or irrigated with Se-rich waters (Broadley et al., 2006; Zhu et al., 2009; Bañuelos et al., 2017; Hossain et al., 2021). For example, using Se-laden drainage water as an alternative source of water in drought-stricken California, is also a source of Se for Se biofortification strategies. However, identifying crops that are salt and B tolerant, is a prerequisite for any water reuse strategy implemented in the westside of Central California, because of the abundance of geogenic sources of salts.

In this regard, halophytic plants may be an alternative salt tolerant plant species to consider that can be cultivated on saline soil and utilize saline waters (Galvani, 2007; Flowers and Colmer, 2008; Rozema and Flowers, 2008; Díaz et al., 2013). In the westside of Central California, other salt-tolerant forage species, i.e., atriplex, have been sustainably irrigated with Se-enriched saline water to produce Se-enriched annual forage (Watson, 1990; Watson and O'Leary, 1993). Although many halophytic species have primarily been utilized in animal forage (Norman et al., 2013; Attia-Ismail, 2018; Marinoni et al., 2019), a few halophytic plants have had a place in the diet of people around the world (Watson, 1990; Watson and O'Leary, 1993; Panta et al., 2014). In contrast to utilizing halophytes as forage, the halophytic plant, *Salsola soda*, 'agretti', native to saline soil in the coastal regions of the Mediterranean basin, is commonly consumed in many parts of Southern Europe, i.e., Italy (Colla et al., 2006; Minuto et al., 2011). The plant is farmed as a vegetable and in folk medicine, *Salsola* species, was used to treat hypertension, constipation and inflammation (Tundis et al., 2009; Iannuzzi et al., 2020).

In 2014, initial greenhouse work by Centofanti and Bañuelos (2015) evaluated agretti's ability to tolerate irrigation water with high salinity, B, and Se when grown in saline soils. They showed that *Salsola soda* can grow in saline (EC > 10 dS m^{-1}) and B-laden soils (10 mg/L^{-1}) and tolerate irrigation with saline and B and Se rich water (EC of 3 dS m^{-1}, 4 mg B L^{-1} and 0.1 mg Se L^{-1}). Moreover, the plant extracted and accumulated Se and Na. In this regard, concentrations of tissue Se ranged from 3 to

5 mg kg^{-1} DW and concentrations of plant Na were as high as 8% DW under high saline growing conditions. Agretti's ability to extract selected ions, i.e., Se and Na, under saline conditions suggests that the plant may be a candidate for Se biofortification and may also be useful for biologically managing soluble Se and Na added to soils when using saline waters originating from the westside of the SJV.

Based upon the initial research conducted by Centofanti and Bañuelos (2015) on the impact of salinity and B on the accumulation of Se, the current multi-year field Se biofortification study was established on growing agretti under saline soil and saline irrigation conditions. To the best of our knowledge, there have been no investigations on growing agretti as a Se-biofortified crop under saline irrigated field conditions in the westside of the SJV in California. Therefore, the goal of multi-year field study was to determine if agretti can be considered an alternative Se-biofortification crop for growing under organic-like conditions in saline/B and Se laden soils and irrigated with either saline water or low-saline water. We will identify the impact of saline and low-saline waters applied at different rates on agretti's growth, Se accumulation (including chemical forms of Se accumulated) when producing a Se-biofortified product under saline growing conditions. Results of this study should provide evidence for promoting agretti as an alternative Se-enriched crop grown with either Se-laden saline drainage water or low-saline water in saline and Se-rich soils of the westside of the SJV or in other similar geological regions containing Se, salinity, and, e.g., Colorado, China.

Materials and methods

Field experiment

The saline, B- and Se-laden microplots were established at Red Rock Ranch (RRR), Five Points, CA (36°22′59.73″ N and 120°13′44.94″ W). The soil is classified as an Oxalis silty clay loam (fine montmorillonitic, thermic in Pachic Haploxeral with a well-developed salinity profile). Soil salinity at the field site soil ranged from 7 to 16 dS m^{-1}, soluble B from 10 to 18 mg L^{-1}, and soluble Se from 0.175 to 0.500 mg L^{-1}, respectively. The multi-year study took place in 2016, 2017, 2018, and 2021 (described below). The field sites for plantings in 2016 and in 2017 consisted of 18 raised planting beds (30 m long and 180 cm wide), respectively, while 18 raised planting beds (30 m long and 45 cm wide) were used in planting in 2018. In the 2021 planting, the beds consisted of eighteen 15 m long and 45 cm wide (**Supplementary Figure 1**). Over the course of this four-year field study, agretti *(Salsoda soda* L.*)* seeds were germinated in seedling trays under greenhouse conditions prior to being transplanted into the field microplots. Greenhouse conditions for growing seeds were generally as follows: day/night temperatures 26/20°C, 16h photo period, 20-30% relative humidity of ambient air, and an average daily 500-800 μmol photons m^{-2} s^{-2} light intensity. Agretti was transplanted as 3-4 weeks old plantlets in the field sites in 2016, 2017, 2018, and direct-seeded in 2021 (described later). For planting, thirty-day old plants were transplanted 20 cm apart in two rows, which were 50 cm apart from each other in year 2016, 2017 and 2018. In 2021, we also did direct seeding (described below) with 1 cm distance between seeds on 9 beds (15 m long and 45 cm wide). In years 2019 and 2020, germination rates were too poor, and consequently no planting occurred at the field sites. During this multi-year study, we observed that for all tested years, agretti's seed viability was very poor beyond a storage period of three months. Hence, there was only one growing season per year because seed germination was too low.

For each planting, two soil samples were collected to a depth of 0-30 and 30-60 cm in each sub-plot (three one-meter sub-plots randomly located per bed) at preplant and at harvest for each respective planting and composited, respectively, for each bed. **Table 1** shows soil chemical properties, including acid extractable Se concentrations for three different soil depths (0–30, 30–60, 60–90 cm) at pre-planting for each growing year. Soil samples were processed, as described later. Plants were field-grown under organic- like conditions without the use of synthetic chemicals, i.e., pesticides, herbicides, or fertilizers. Although the growing conditions were not certified as organic, because of the environmentally fragile conditions (water scarcity and saline soils), growers at Red Rock Ranch are required to follow California state guidelines similar to organic operation (CDFA, 2020).

Plants were grown during four different growing seasons each year due to the variation in seed viability and germination rates amongst the four years. The first planting occurred from 11 July to 21 August 2016; the second planting occurred from 23 May to 24 July 2017; the third planting occurred from 16 April to 25 June 2018; and the fourth planting occurred from 24 February to 26 April 2021. Plantings in 2016 and 2017 took place on the same field plot, while planting in 2018 and 2021 took place on a field site adjacent to field site used in the earlier plantings.

Irrigation treatments

A surface-drip irrigation system was installed consisting of one in-line turbulent flow emitter per bed with an emitter spacing of 0.45 m and a flow rate of 4 L/h on the field site. Low-saline water (EC < 1.0 dS m^{-1}) was sprinkled irrigated and applied at time of transplanting to promote initial establishment of plants. The amount of total irrigation water applied to microplots was based on rates of 100% evapotranspiration (Et_o) (treatment "High"), 75% Et_o (treatment "Medium"), and 50% of Et_o (treatment "Low"), respectively. Irrigation amounts were determined by multiplying the average potential

TABLE 1 Soil chemical properties at field sites at pre-planting for growing seasons in 2016, 2017, 2018, and 2021, respectively. Values represent average (n = 3) ± SD.

Year	Depth cm	pH	EC mS/cm	Water extractable mg/L Cl	B	Ca	K	Mg	Na	S	Se	Acid Extractable[§] Se mg/kg DW
2016	0–30	7.99 ± 0.09	8.07 ± 1.59	510 ± 146	10.2 ± 2.2	488 ± 21	17.9 ± 2.4	60 ± 5	1614 ± 457	1390 ± 240	0.06 ± 0.03	1.6 ± 0.1
	30–60	8.14 ± 0.11	13.6 ± 0.95	937 ± 170	17.2 ± 1.4	466 ± 15	17.7 ± 1.4	73 ± 3	3372 ± 254	2365 ± 121	0.22 ± 0.07	1.5 ± 0.2
	60–90	8.15 ± 0.10	16.0 ± 1.25	1237 ± 187	18.7 ± 1.5	463 ± 14	19.6 ± 2.0	78 ± 8	4053 ± 346	2679 ± 188	0.29 ± 0.06	1.2 ± 0.1
2017	0–30	7.99 ± 0.21	6.99 ± 1.61	277 ± 145	9.2 ± 3.4	485 ± 15	13.0 ± 4.7	54 ± 8	1282 ± 410	1246 ± 271	0.12 ± 0.06	1.9 ± 0.2
	30–60	8.27 ± 0.11	13.0 ± 1.39	827 ± 246	18.6 ± 2.5	488 ± 29	11.6 ± 2.8	72 ± 5	2894 ± 336	2231 ± 196	0.34 ± 0.09	1.7 ± 0.4
	60–90	8.24 ± 0.17	16.5 ± 1.75	1360 ± 415	21.4 ± 3.6	504 ± 37	12.1 ± 4.3	85 ± 12	3766 ± 442	2731 ± 246	0.43 ± 0.16	1.5 ± 0.2
2018	0–30	8.12 ± 0.04	8.47 ± 1.32	529 ± 267	12.0 ± 2.1	316 ± 72	19.1 ± 4.1	102 ± 18	1743 ± 397	1212 ± 160	0.52 ± 0.13	3.4 ± 0.4
	30–60	8.29 ± 0.06	12.5 ± 0.96	951 ± 145	13.9 ± 1.1	237 ± 73	14.1 ± 6.0	128 ± 11	3045 ± 317	1871 ± 203	1.14 ± 0.25	2.2 ± 0.4
	60–90	8.30 ± 0.07	14.0 ± 0.98	1261 ± 283	12.4 ± 1.1	180 ± 64	14.8 ± 5.1	137 ± 13	3558 ± 333	2048 ± 222	0.85 ± 0.24	1.5 ± 0.2
2021	0–30	7.88 ± 0.06	10.33 ± 2.71	1582 ± 574	12.2 ± 4.0	563 ± 52	22.4 ± 7.9	110 ± 26	1825 ± 655	1122 ± 237	0.44 ± 0.12	1.1 ± 0.2
	30–60	8.26 ± 0.11	13.0 ± 0.87	1008 ± 152	16.0 ± 1.2	397 ± 42	11.3 ± 2.8	91 ± 9	3274 ± 281	2156 ± 192	0.56 ± 0.14	1.8 ± 0.2
	60–90	8.23 ± 0.91	15.5 ± 1.09	1286 ± 236	17.4 ± 1.3	449 ± 38	15.5 ± 3.2	100 ± 16	3792 ± 401	2486 ± 248	0.52 ± 0.09	1.4 ± 0.1

[§]Acid extractable Se in 2021 were estimated based upon pervious planting years.

TABLE 2 Total amounts of either low-saline or saline water applied at different rates (Et_o %) to microplots for all four years for planting seasons in 2016, 2017, 2018, and 2021, respectively.

Year of planting	Days of irrigation	Treatment (Et_o%) 100%	75%	50%	Precipitation
		mm			
2016	40	263	203	144	0
2017	62	485	363	249	1
2018	70	430	308	217	7
2021[†]	56	201	149	99	29

[†]Direct-seed planting took place in February of 2021 (see section materials and methods).

evapotranspiration (ET_o) data recorded by CIMIS station #2 (California Irrigation Management Information System at Five Points/UC Westside Field Station) by forage crop coefficient (K_c), averaging 0.35 (early season) to 1.15 (mid-season) to 0.75 (end of season). Crop coefficients were adjusted according to their respective growing season for each respective planting. Information on the total amount of water applied per irrigation water treatment (high, medium and low was based on ET_o) is shown in Table 2. The experimental site was a completely randomized block design. Each irrigation treatment was replicated six times, with a replicate consisting of one bed (already described) (Supplementary Figure 1). Two types of water quality were used for irrigation for the multi-year study: low-saline water and saline water. Low-saline water solely was used to irrigate agretti in 2016 and 2017, and saline water only was used to irrigate agretti in 2018 and 2021. The saline water was collected from furrow irrigated field sites adjacent to the microplots. This source of water was collected and stored in a drainage pond reservoir adjacent to test field site. The saline water was then pumped, filtered, and utilized in the agretti field plot with the surface drip irrigation system. Saline water composition used for irrigation on the microplots generally had salinity levels ranging from 3 to 8 dS m^{-1}, 4–8 mg B L^{-1} and 0.12–0.25 mg Se L^{-1} while low-saline water had salinity levels ranging from 0.2 to 0.7 dS m^{-1}, 1 mg B L^{-1} and 0.02 mg Se^{-1} (water quality characteristics are shown in Table 3).

Harvest

Plants were grown for 40 d, 61 d, 69 d, and 50 d[1] for plantings in 2016, 2017, 2018, and 2021, respectively. Harvest for each year generally occurred after we visually determined that "agretti" shoots were still young and

1 Fifty days was selected for harvest in 2021 because the plants looked more consumers' friendly (e.g., young tender and soft tissues). These characteristics were more noticeable in direct-seeded plants.

TABLE 3 Chemical characteristics of low-saline water and saline water applied to microplots for growing seasons in 2016, 2017, 2018, and 2021, respectively. Values represent average (n = 3) ± SD of water samples collected through respective growing season.

Water quality	Year	pH	EC	Cl	B	Ca	K	Mg	Na	S	Se
			mS/cm	-mg/L							
Low-saline‡	2016	7.63 ± 0.12	2.95 ± 0.6	554 ± 66	3.5 ± 0.7	85 ± 31	2.1 ± 1.0	4.7 ± 2.2	624 ± 163	249 ± 57	0.06 ± 0.02
	2017	7.79 ± 0.5	3.43 ± 1.3	517 ± 84	4.4 ± 1.7	71 ± 24	2.4 ± 0.8	3.1 ± 1.4	761 ± 161	377 ± 266	0.08 ± 0.03
Saline	2018	8.37 ± 0.5	9.25 ± 2.4	1257 ± 351	16.3 ± 5.8	427 ± 137	5.8 ± 2.1	108 ± 39	1606 ± 505	903 ± 305	0.35 ± 0.14
	2021	8.01 ± 0.2	5.19 ± 0.3	1244 ± 240	5.1 ± 0.2	134 ± 42	5.6 ± 1.0	48 ± 6	770 ± 75	199 ± 11 5	0.01 ± 0.01

‡Waters were also tested for biological and pathogenic activities.

tender (preferred for human consumption), irrespective of water treatment. Above-ground young vegetative growth was cut 1 cm above the soil at soil sampling sites located in sub-plots within each bed. For analyzing data, plant biomass and yield of the single cuttings were measured on the three one-meter sub-plots (**Supplementary Figure 1**) randomly selected for each respective water treatment on each bed. Shoots samples were washed in deionized water, weighed, dried at 55–65^{o}C for three days, weighed again, and ground with the Udy Cyclone Mill with a 1.0 mm screen. Twenty grams composite sub-samples of freshly harvested agretti from each respective planting were placed in chests filled with ice, transported to laboratory, and stored at −80°C for future analyses on Se speciation and total phenolics (described later). The other remaining plants on field site were harvested and donated to restaurants and farmers' markets, which showed a strong interest in this new Se enriched halophytic vegetable (its potential marketability is discussed later). After harvest, soil samples were again collected at the same sub-plot locations sampled prior to transplanting, as already described. They were dried and processed, as already described for shoot material.

Selenium extraction: Soluble and protease

To determine Se speciation in agretti shoot samples, sub-samples store at - 80°C were retrieved. Due to unexpected power outages and loss of electricity, all sample stored at -80°C were lost, except samples collected from planting (transplanted and direct-seeded) in 2021. For Se speciation the samples were processed as follows: methanol chloroform water (MCW) solvent extraction (described as "soluble" throughout text) and MCW enzymatic digest (with protease) were used to separate the soluble Se compounds (non-protein bound) and insoluble compounds (protein bound) for identification and quantification. The MCW extraction used 1 g of freeze-dried ground and sieved tissue sample added to 40 mL glass vials with a Teflon cap and separated in two sets of replicates (soluble and protease). Fifty mg of protease from Streptomyces griseus Type XIV (Sigma-Aldrich) was added to the protease replicates (Montes-Bayón et al., 2002), which hydrolizes peptide bonds, releasing Se amino acids into solution. Next, 10 mL of ultrapure water at room temperature were added to these vials containing protease. The other set of samples (soluble) received 17 mL of methanol (Optima grade) and contained no protease. The samples were vortexed, and the protease sample set was incubated in a shaker for 20 h at 37°C, while the methanol only sample set was placed overnight at 4°C. After digestion, 17 mL of methanol were added to the protease samples (to denature the protease enzyme and stop enzymatic activity), and 10 mL of ultrapure water were added to the soluble digested methanol extractions. Each tube was vortexed multiple times and refrigerated overnight at 4°C. Following this, 8.5 mL of chloroform (Optimal grade) were added to all vials and capped, shaken vigorously, and refrigerated at 4°C overnight until the tissue was fully extracted, and the upper aqueous (methanol-water) phase had fully partitioned from the chloroform phase. The upper aqueous (methanol-water) containing the extracted Se compounds was removed and transferred to a centrifuge tube. One quarter of the aqueous (methanol-water) phase was then pipetted into 50 mL ICP digestion tubes for drying, acid digestion, and analysis of total aqueous Se by ICP-MS (described later). The fully extracted tissue remaining in the chloroform phase was then dried, acid digested, and analyzed for total Se by ICP-MS. The Se extraction efficiency (80%) in the aqueous phase (soluble and protease extracts) was calculated from these ICP-MS results as: (total Se in methanol-water phase) / [(total Se in methanol-water phase) + (total Se in chloroform phase)] × 100. The remaining aqueous (methanol-water) phase was dried in vacuum at -140°C by refrigerated centrifugal speed vacuum (Labconco CentriVap Concentrator), re-suspended to 2.5 mL with ultrapure water, and stored in a −80°C freezer. Final clean-up of the concentrate used Waters Sep-Pak Classic C18 cartridge (360 mg 55–105 μm). Each cartridge was cleaned by flushing 10 mL of methanol and 5 mL ultrapure water in succession. The 2.5 mL concentrates were thawed, vortexed, and 11 μL of 88 % formic acid (ACS grade, Fisher Chemical) were added prior to being transferred by disposable Pasture

pipette to the Sep-Pak. The column was loaded with the sample, which was pushed through, and soluble residual was eluted with 3 mL methanol. The total eluent (methanol-water) was collected into a 50 mL conical tube and then dried completely using refrigerated centrifugal speed vacuum. This dry extract pellet was then re-suspended in 1.5 mL ultrapure water and centrifuged in Corning Costar Spin-X centrifuge tube filters (0.22 μm at 10,000 rpm). The filtered samples were then transferred into Agilent 2 mL screw top glass vials with septa and frozen until SAX-HPLC-ICP-MS analysis.

Selenium speciation and total Se analyses

The Se speciation analysis (organic and inorganic Se) of the soluble and protease extracts from agretti is described in detail by Bañuelos et al. (2012). Selenium speciation analyses used an Agilent 1200 HPLC equipped with a Hamilton PRP-X100 strong anion exchange column (10 μm particle size 250 mm length and 4.1 mm internal diameter) coupled to the Agilent 7500 CX ICP-MS (SAX-HPLC-ICP-MS). The ICP-MS was equipped with a quadrupole detector and an Octopole Reaction System (ORS) utilizing hydrogen as a cell gas (5.5 mL/min) to minimize Se polyatomic interferences. Dried ground agretti was analyzed for total Se concentrations by Agilent 7500 CX ICP-MS (Agilent Technologies Santa Clara, USA) and other elements with the inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian Vista-Pro Santa Clara, CA, USA) after wet-acid digestion with HNO_3, and H_2O_2, and HCl. Generally, for Se speciation a single analysis (30 μL injection) was conducted for each of the broths, soluble, and protease extract replicates (n = 3). Chromatographic separation of Se was achieved with an isocratic mobile phase of 5 mM ammonium citrate buffer (pH 5.2) with 2% methanol at flow rate of 1 mL/min. The two instruments (Agilent 1200 HPLC and Agilent 7500 CX ICP-MS) were integrated through Agilent Chemstation software with chromatographic data analysis. The retention times of Se-78 containing peaks were monitored using the ICP-MS and directly compared to the authentic standard (listed below), retention times, and secondary confirmation by spiking samples with standards to account for any matrix induced changes to the chromatographic analysis, as described by Bañuelos et al. (2012). The SAX-HPLC-ICP-MS standards utilized included sodium selenate (Na_2SeO_4), sodium selenite (Na_2SeO_3), SeMet, and $SeCys_2$ (all purchased from Sigma-Aldrich, St. Louis, MO). Additionally, methyl-selenocysteine (MeSeCys), selenocystathionine (SeCyst), and γ-glutamyl-methyl-selenocysteine (γ-gluMeSeCys) were all purchased from Pharma Se.

Quality control for Se and Se speciation

The National Institute of Standards and Technology (NIST) wheat flour (SRM 1567a) was used as the standardized quality control for wet-acid digestion (total Se concentration) and Se speciation extraction (SeMet, $SeCys_2$) content in plant material. The SRM 1567a was utilized as an internal control in the MCW extraction to account for any changes in the protease XIV efficacy and other factors during extraction process. The total Se recovery rates were over 94 % for the wheat flour standard, which has a Se concentration of 1.1 ± 0.2 μg Se/g DW, with a method detection limit of 50 μg Se/g DW. The selenoamino acid content in SRM 1567a consisted of 92% SeMet and 6% $SeCys_2$. The NIST wheat flour standard was always included in triplicate with each plant powder and respective agretti sample. Overall, Se speciation extraction efficacy, including MCW (soluble; free and unbound Se) and protease extractable Se (protein bound Se) was at least 80% for agretti, and wheat standard matrixes. The extraction and quality control measures are documented in detail (Bañuelos et al., 2012).

Total phenolics

Total phenolic concentrations were measured in stored agretti samples from planting 2021 (described earlier) according to (Singleton et al., 1998) using the Folin-Ciocalteu reagent assay. Absorbance was measured at 756 nm using a Spectra Max plus 384 spectrophotometer (Molecular Devices, Sunnydale, CA). Total phenols concentration was standardized against gallic acid (GA) and expressed as milligram of gallic acid equivalents (GAE) per L of fruit juice. The linearity range for this assay was determined as 50-250 mg/L GA, giving absorbance range of 0.5–2.55 AU. The total phenolic analyses is used as an indicator of plant stress.

Statistical analysis

All data were analyzed using Sigmaplot version 14.5. We tested significance and pairwise comparison amongst the irrigation treatment (levels of Et_o) in each planting year because plants were grown during four different growing seasons. Significance was set at the 5% level. Data have been log transformed when they were not normally distributed. There were about 5–8% outliers that have been averaged out with the non-outliers within the same replication group. Statistical data analysis was performed with Gretl [Gnu

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Regression, Econometrics and Time-series Library, (Baiocchi and Distaso, 2003)].

Results

Plant growth

In this study, no plant toxicity symptoms were observed for any planting; irrespective of water quality applied or year of planting. Results of fresh and dry weight of agretti are shown in Figure 1 for all four years. Fresh weight yields were highest in 2017 (plants were irrigated with low-saline water and growing season was May thru July) compared to the different seasons in other planting years. Overall, fresh biomass was significantly higher when the plants received water of either quality at 100% Et_o compared to 75 and 50% Et_o for all plantings, except for 2016, when yield at 100 and 75% Et_o were similar. In 2016, 2017, and 2018, dry weight was not affected by the rate of irrigation water applied, and the values of dry weight were comparable across treatments (Figure 1). In 2021, the dry weight was significantly lower than in previous years (Note: 2021, yields are presented from plants that were direct seeded). The amount of irrigation water applied affected dry weight, causing a significantly lower dry weight at 50% Et_o compared to 100 and 75% Et_o treatments.

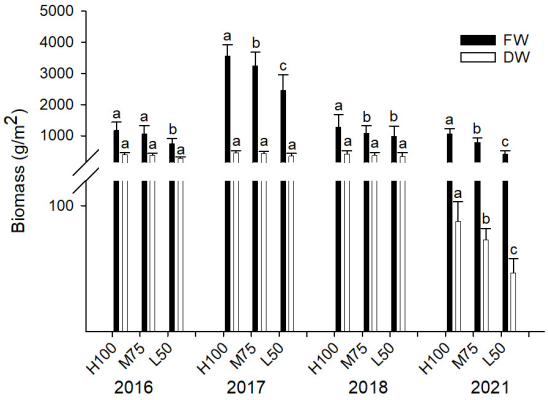

FIGURE 1
Fresh and dry weight of plant biomass in agretti grown for 4 years on saline, B- and Se-laden soil and irrigated with either low-saline water (2016 and 2017) or saline water (2018 and 2021). Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50% Et_o) as described in Table 3. Values represent averages (n = 12 for 100% Et_o, n = 9 for 75% and 50% Et_o for planting in 2016; n = 19 for 100% Et_o, n = 17 for 75% for planting in 2017; n = 18 for planting in 2018, n = 4 for planting in 2021). Values of n varies amongst year because of the variability in seed germination and plantlets survival in the field. and error bars represent standard deviation. Different letters indicate that values differed significantly ($P \leq 0.05$), ns = not significant.

Plant nutrients

Among the plant macronutrients some effects from irrigation rates and quality were observed. Magnesium was significantly higher when plants received 75 and 50 % Et_o in 2021 and in 2018, when plants received 50% Et_o (Supplementary Table 1) with saline water. P was significantly higher in 2016 and 2017, when the plants were irrigated with low-saline water and S was significantly higher in 2017 (when irrigated with low-saline water), and in 2018 when plants were irrigated with saline water (Supplementary Table 1). The micronutrients, Fe, Mn, and Zn were significantly higher in 2021 with irrigation with saline water but Cu was significantly lower (Supplementary Table 2). Fe was higher in 2021 when the plants were irrigated with 75% of Et_o and generally, Cu, Mn, and Zn were only affected by the planting year and not the water treatment (Supplementary Table 2).

Selenium

The concentration of Se in agretti ranged from 0.2 to 0.7 mg/kg DW in 2016 and 2017 with low-saline irrigation water, and 2.1 to 3.6 mg/kg DW in 2018 and 2021, respectively, with saline irrigation water (Figure 2). There was no significant effect of irrigation Et_o% treatment on Se accumulation in shoots for any year, irrespective of water quality. Our results indicate that agretti is able to accumulate high levels of Se in its edible biomass when grown in Se-rich soil and irrigated with either saline or low-saline water. We observed, however, higher concentrations of tissue Se when irrigating with Se-containing saline water.

Figures 3, 4 show concentrations (mg/kg DW) of Cl and Na in harvested dried plant material. Na and Cl concentrations in shoots were >2% for Cl and >5% for Na, indicating that agretti, being a halophyte, accumulates high levels of these salt ions in its shoots, irrespective of saline or low-saline irrigation. The concentration of Cl was significantly higher in the 75 and 50% Et_o treatments compared to 100% Et_o in 2016, 2017, and 2018. In 2021, the concentration of Cl was, however, significantly higher at 100% Et_o compared to 75 and 50% Et_o (Figure 3). There were no significant differences in Na concentrations in shoots across treatments and years, and the concentration of Na was higher in 2021 (8–10% DW) compared to previous years (3–5 % DW) (Figure 4); In 2016 and 2017, the plants were irrigated with low-saline water compared to irrigation with saline water in 2018 and 2021. The different irrigation water quality did not strongly affect Na and Cl accumulation in shoot (Figures 3, 4). Concentrations of both ions were similar in 2016, 2017, and 2018, since the plants were grown in saline soil for all treatments every year, irrespective of water quality applied via irrigation.

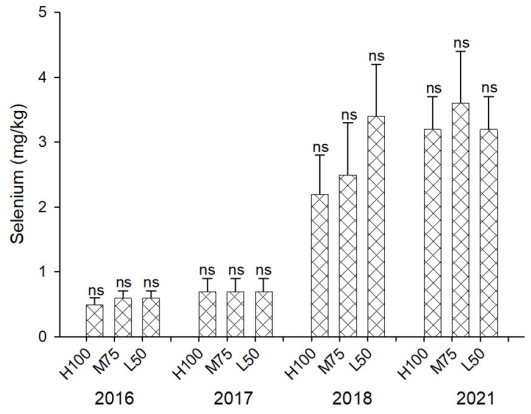

FIGURE 2
Concentration (mg/kg DW) of Se in plants of agretti grown for 4 years on saline, B- and Se-laden soil and irrigated at different rates (Et_o %) with either low-saline water (2016 and 2017) or saline water (2018 and 2021). Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50%Et_o), as described in **Table 3**. Values represent average (n = 12 for 100% Et_o, n = 9 for 75% and 50% Et_o for planting in 2016; n = 19 for 100% Et_o, n = 17 for 75% for planting in 2017; n = 18 for planting in 2018, n = 4 for planting in 2021, direct seeding) and error bars represent standard deviation. There was no significance amongst any treatments.

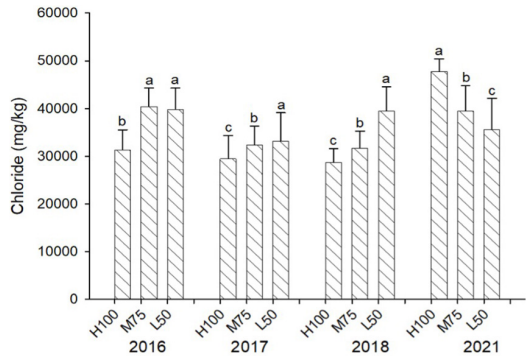

FIGURE 3
Concentration (mg/kg DW) of Cl in plants of agretti grown for 4 years on saline, B- and Se-laden soil and irrigated at different rates (Et_o%) with either low-saline water (2016 and 2017) or saline water (2018 and 2021). Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50% Et_o) as described in **Table 3**. Values represent average (n = 12 for 100% Et_o, n = 9 for 75% and 50% Et_o for planting in 2016; n = 19 for 100% Et_o, n = 17 for 75% for planting in 2017; n = 18 for planting in 2018, n = 4 for planting in 2021, direct seeding) and error bars represent standard deviation. Different letters indicate that values differed significantly ($P \leq 0.05$), ns = not significant.

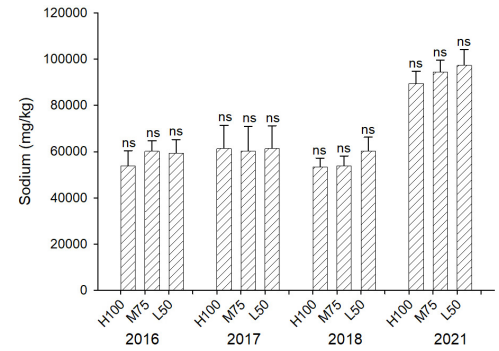

FIGURE 4
Concentration (mg/kg DW) of Na in plants of agretti grown for 4 years on saline, B- and Se-laden soil and irrigated at different rates (Et_o%) with either low-saline water (2016 and 2017) or saline water (2018 and 2021). Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50% Et_o) as described in **Table 3**. Values represent average (n = 12 for 100% Et_o, n = 9 for 75% and 50% Et_o for planting in 2016; n = 19 for 100% Et_o, n = 17 for 75% for planting in 2017; n = 18 for planting in 2018, n = 4 for planting in 2021, direct seeding) and error bars represent standard deviation. Different letters indicate that values differed significantly ($P \leq 0.05$), ns = not significant.

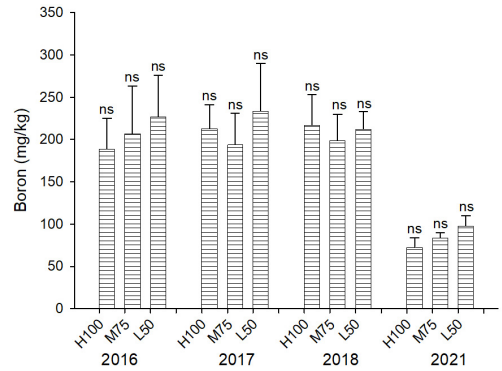

FIGURE 5
Concentration (mg/kg DW) of B in plants of agretti grown for 4 years on saline, B- and Se-laden soil and irrigated at different rates (Et_o%) with either low-saline water (2016 and 2017) or saline water (2018 and 2021). Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50% Et_o) as described in **Table 3**. Values represent average (n = 12 for 100% Et_o, n = 9 for 75% and 50% Et_o for planting in 2016; n = 19 for 100% Et_o, n = 17 for 75% for planting in 2017; n = 18 for planting in 2018, n = 4 for planting in 2021, direct seeding) and error bars represent standard deviation. Different letters indicate that values differed significantly ($P \leq 0.05$), ns = not significant.

Concentrations of B are similar across all water treatments (ET_o) (**Figuref 5**), and the addition at B applied with saline water in 2018 (16 mg B/L) did not result in significantly different plant concentrations of B compared to 2016 and 2017. However, the concentration of B in agretti in 2021 was lower than in the previous years (2016–2018). This indicates that direct-seed planting from seeds may increase the ability of agretti

to tolerate higher B concentrations, if they accumulated lower concentrations of B.

Selenium speciation and total phenolics

Unfortunately, only tissues samples collected in 2021 were analyzed for Se speciation and total phenolics (as previously mentioned stored agretti samples at –80°C from 2017, 2018, and 2019 were lost) due to electrical power outage. **Figure 6** shows Se speciation in agretti planted directly by seed in 2021. Irrespective of water treatment, SeMet was always the predominate Se species (between 60 and 70%), followed by SeO_4^{-2} (20-32%) and then $SeCys_2$ (2–5%). There were no significant effects of irrigation treatment (Et_o%) for all Se species, except for $SeCys_2$, which showed a significantly higher concentration with high irrigation treatment (100% Et_o > 75% Et_o > 50% Et_o).

Limited phenolic data (as described in the section materials and methods) showed values that did not exhibit any significant stress (indicated by total phenolic content) for agretti grown as direct seeded for any irrigation treatments. Total phenolics ranged from 180 GA mg/L (at highest treatment of 100% Et_o) to a high of 257 GA mg/L (at lowest water treatment of 50% Et_o).

Soil analyses

Soluble soil chemical properties are shown at postharvest for all four years in **Table 4**. Averaged over four years, soil salinity ranged from a low of 3 to a high of 14 dS m^{-1} (**Table 4**), soluble B ranged from a low of 3 to a high of 19 mg/L, and soluble Se ranged from a low of 30 to a high of >1000 μg/L. These levels of salinity and B are considered toxic to most agronomic crops (Grieve et al., 2011). Soil salinity, soluble B and Se concentrations were significantly higher at the deeper depths (30–60 cm) after harvest. Generally. Soil EC was greater with 50 and 75% Et_o treatments. Irrigation at 100% Et_o likely induced some leaching of salinity compared to lower Et_o treatments.

Discussion

This study is a first field investigation on the feasibility of growing agretti under organic- like growing conditions as a Se-biofortified crop under saline and B-irrigated field conditions in the westside of Central California. Previous studies have identified agretti as a potential halophyte crop for growing in saline conditions (Colla et al., 2006; Calone et al., 2021), as well as in in saline/Se-rich soils and irrigating with Se-enriched water under greenhouse conditions (Centofanti and Bañuelos, 2015; Zhu et al., 2019). In this field study, agretti was grown on Se-rich soil and irrigated with either low -saline water or with saline water naturally enriched with Se and B at different irrigation rates (% of ET_o). The natural occurrence of Se in the soil and the additional contribution of Se via saline water application, resulted in a high accumulation of Se, irrespective of water application rate. Growing agretti in a Se rich soil and irrigating with saline water appears to be a natural strategy for more effectively producing Se biofortified agretti under these arid growing conditions. The mean concentration of tissue Se in agretti with low-saline irrigation water was 640 μg Se kg^{-1} DW and 2940 μg Se kg^{-1} DW with saline irrigation. If a serving portion (100 g fresh agretti material, corresponding to about 30 g DW) was consumed, then agretti (with tissue Se concentrations presented in **Figure 2**) could provide 19.2 μg Se/serving when harvested from low-saline irrigation and 88.2 μg Se/serving when harvested from saline irrigation, on these saline/Se-laden soils, respectively. The average required level of Se in the human diet is 50–55 μg/day Se (Gupta, 2020). Although the window between toxicity and deficiency is narrow (∼ 400 μg Se/day vs ∼ 40 μg Se/day), Se deficiency is more widespread than Se toxicity (Coppinger and Diamond, 2001; Broadley et al., 2006; White and Broadley, 2009). Since plants are the main source of Se for most humans and livestock across the world, we have demonstrated that Se biofortification is possible with agretti when irrigated with low-saline or saline water in the westside of Central California.

Our results do not show a clear trend of increased Se accumulation in agretti under water deficit irrigation, i.e., 50% Et_o. Thus, it appears that water application rate of either low-saline or saline water does not significantly affect Se accumulation in agretti grown in saline soils. Selenium accumulation in plants grown under saline growing conditions may be advantageous for the plant, because Se accumulation in plants may affect other physiological processes within plants related to increasing salt tolerance. In this regard, others have shown that accumulated Se in plants participates in antioxidant defense systems and it may enhance tolerance to abiotic stresses (Andrade et al., 2018), such as water deficit or excessive salinity. Djanaguiraman et al. (2005) reported that Se may play an important role in the adjustment of plant water status under drought stress and improve plant–water relations by lowering the osmotic potential of seedlings growing under water stress (Hartikainen, 2005; Nawaz et al., 2012). In addition to the role of Se potentially enhancing stress tolerance, halophytes, including Salsoda species, are already adaptive to tolerating high salinity (Shuyskaya et al., 2017; Hasanuzzaman et al., 2019; Mukhtar et al., 2019). Hence, the accumulation of Se may provide agretti with additional tolerance to high salinity.

The speciation of Se in agretti shoots in 2021 showed that for all irrigation treatments, SeMet was the predominant selenoamino acid, irrespective of planting method or any water treatment (i.e., application rate). Moreover, water application (ET_o%) had no effect on Se speciation, except for $SeCys_2$. Inexplicable effects from irrigation treatment

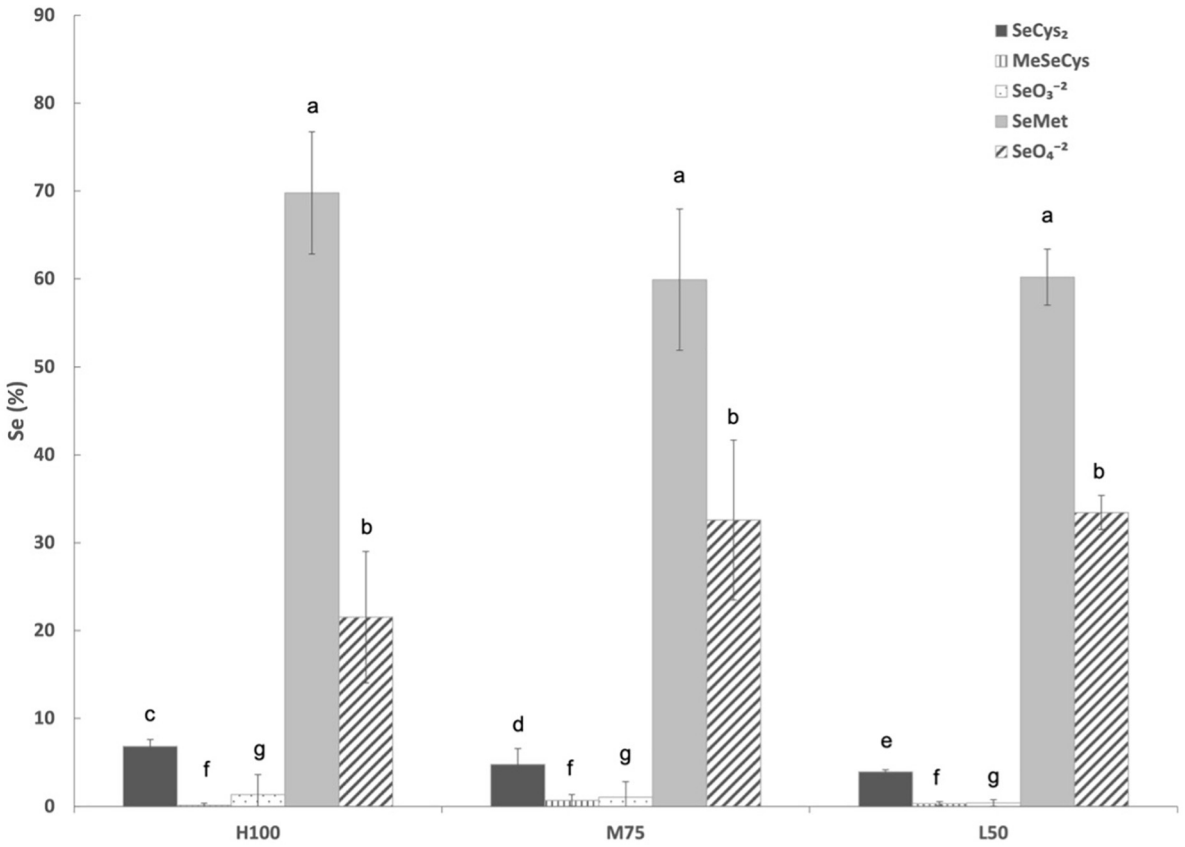

FIGURE 6
Selenium speciation in direct seeded agretti grown in 2021 and irrigated with saline waters at different rates. Statistical difference was performed to analyze the effect of irrigation levels within each planting year (H100 = high 100% Et_o, M75 = medium 75% Et_o, and L50 = low 50% Et_o) as described in **Table 3**. Values represent the mean (n = 3) and error bars represent standard deviation. Different letters indicate that values differed significantly ($P \leq 0.05$), ns = not significant. $SeCys_2$: Selenocysteine; MeSeCys: Methylselenocysteine; SeO_3^{-2}: Selenite; SeMet: Selenomethionine; SeO_4^{-2}: Selenate.

were observed in $SeCys_2$. We are currently investigating this irrigation effect on Se accumulation in other crops, e.g., tomatoes, to determine if irrigation with saline water influences Se speciation, a component important to understand in any biofortification strategy. Like most Se speciation identified in non-Se accumulator plant species, SeMet Is the predominant selenoamino acid in agretti biofortified with Se under natural growing conditions. Thus, consumption of Se-enriched agretti should increase Se intake by consumers.

In addition to being a potential Se-biofortification crop, agretti confirmed its salt tolerance (Calone et al., 2021) as well as B tolerance. Fresh and dry weight were not strongly affected by the presence of B and salts in the soil and saline irrigation water. The reasons for the higher fresh weight yields in 2017 may be related to combination of factors such as the planting date (May 23 to July 24) and the consequent different air temperatures (**Supplementary Figure 2**), the amount of water applied in each year, and the effect of irrigation water quality (low-saline versus saline water). Plant stress, also indicated by changes in total phenolic content, did not significantly differ among water treatments (Et_o %) in 2021. In this study, yields difference among the plants grown are not an accurate indication of salt stress, since harvest time for each respective growing season was virtually determined by the apparent tenderness of the edible shoot. This parameter of harvesting only tender shoots is important because consumers prefer eating young shoots and not shoots from older plants that are slightly more woody (Lone, unpublished). Consequently, yields are controlled strongly by the growers' self-determination of harvest date. We also observed in 2021 that planting directly by seed resulted in a more tender shoot (less stem-like material) a physical characteristic that is more desirable for human consumption, especially for consumers in the Mediterranean region (Renna and Gonnella, 2020; Lombardi et al., 2022). Importantly there was no wood-like stem, which is common physical trait when harvesting transplanted agretti.

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TABLE 4 Soil concentration of Cl, Na, B, and Se, and levels of EC at field site irrigated with different rates (irrigation treatment Et_o) in 4 years at post-harvest of agretti at two soil depths. Values represent average (n = 5) ± SD.

Year		Soil depth	EC	Cl	B	Na	Se	Se
			Water extractable					Acid extractable[§]
	% Et_o	cm	mS/cm	————————-mg/L———————–				mg/kg
2016[+]	100	0–30	6.3 ± 1.3	266 ± 82	6.7 ± 2.1	1101 ± 356	0.06 ± 0.01	1.9 ± 0.2
	100	30–60	13.6 ± 1.4	916 ± 230	18.0 ± 3.4	3117 ± 317	0.24 ± 0.07	1.9 ± 0.3
	75	0–30	7.6 ± 1.6	429 ± 152	10.5 ± 2.5	1453 ± 458	0.09 ± 0.05	1.9 ± 0.1
	75	30–60	14.0 ± 1.4	1049 ± 363	18.2 ± 2.4	3185 ± 380	0.29 ± 0.15	1.8 ± 0.2
	50	0–30	8.86 ± 2.5	521 ± 285	11.2 ± 3.1	1808 ± 705	0.11 ± 0.08	1.8 ± 0.2
	50	30–60	12.9 ± 3.5	903 ± 389	17.9 ± 2.9	2933 ± 964	0.22 ± 0.10	1.7 ± 0.2
2017[+]	100	0–30	4.6 ± 1.4	71 ± 44	5.3 ± 3.4	606 ± 340	0.03 ± 0.02	1.7 ± 0.1
	100	30–60	10.3 ± 1.7	312 ± 212	16.4 ± 3.1	2123 ± 461	0.12 ± 0.07	1.6 ± 0.1
	75	0–30	3.8 ± 0.5	54 ± 34	3.4 ± 1.2	430 ± 132	0.03 ± 0.01	1.7 ± 0.2
	75	30–60	10.5 ± 1.6	335 ± 167	15.6 ± 2.9	2253 ± 447	0.12 ± 0.07	1.6 ± 0.3
	50	0–30	4.74 ± 1.0	80 ± 48	5.0 ± 2.0	662 ± 224	0.03 ± 0.02	1.7 ± 0.2
	50	30–60	12.5 ± 2.2	602 ± 238	19.7 ± 4.0	2759 ± 605	0.2 ± 0.11	1.7 ± 0.2
2018[‡]	100	0–30	8.1 ± 1.9	441 ± 227	11.9 ± 3.9	1543 ± 588	0.27 ± 0.20	3.4 ± 0.3
	100	30–60	12.0 ± 1.5	779 ± 339	16.3 ± 1.7	2726 ± 511	0.89 ± 0.66	2.0 ± 0.4
	75	0–30	9.7 ± 3.1	665 ± 516	14.0 ± 3.9	1959 ± 940	0.34 ± 0.26	2.8 ± 0.6
	75	30–60	13.0 ± 3.6	979 ± 718	16.3 ± 3.3	2891 ± 918	0.8 ± 0.51	1.9 ± 0.1
	50	0–30	9.0 ± 1.0	537 ± 206	14.3 ± 2.2	1709 ± 291	0.32 ± 0.22	3.2 ± 0.3
	50	30–60	14.5 ± 1.6	1345 ± 303	18.0 ± 2.9	3272 ± 539	1.32 ± 0.21	2.5 ± 0.6
2021[‡]	100	0–30	8.97 ± 1.4	834 ± 187	11.2 ± 1.5	1552 ± 335	0.23 ± 0.04	2.3 ± 0.2
	100	30–60	14.43 ± 2.4	1343 ± 420	16.9 ± 2.0	2930 ± 655	0.65 ± 0.07	1.8 ± 0.1
	75	0–30	9.20 ± 0.86	858 ± 128	12.6 ± 2.7	18.52 ± 366	0.22 ± 0.08	2.1 ± 0.2
	75	30–60	12.52 ± 1.8	1122 ± 105	15.9 ± 2.0	2417 ± 457	0.53 ± 0.09	1.7 ± 0.1
	50	0–30	9.77 ± 1.0	959 ± 170	13.7 ± 1.1	1684 ± 280	0.28 ± 0.13	2.2 ± 0.2
	50	30–60	13.36 ± 0.78	1203 ± 106	15.7 ± 2.1	2630 ± 226	0.79 ± 0.16	2.0 ± 0.1

[+] Values represent irrigated with low-saline water.
[‡] Values represent irrigated with saline water.
[§] Acid extractable Se in 2021 were estimated based upon pervious planting years.

Consequently, there were more pronounced differences fresh and dry weight biomass in 2021. This result is likely because plants grown by direct seeding in 2021 contained a higher water content in shoots- there was less stem and more agretti-like leaves.

Levels of accumulated B in shoot of agretti were comparable to those reported by Zhu et al. (2019), who grew agretti in hydroponic system under controlled conditions with solution B concentrations similar to those applied in this study with saline water. In our field study, B concentration in shoots was lower in 2021 compared to previous years. This effect is likely due to high salinity inhibition on inhibiting B uptake (shown in **Figure 4** in 2021). Others have observed this effect of salinity on B uptake in other crops (Yermiyahu et al., 2008; Zhu and Bañuelos, 2016). However, direct-seeded agretti versus transplanted agretti may have inexplicitly also played a role in restricting B accumulation in the agretti shoots; hence enhancing agretti's B tolerance. Thus, our results indicate that direct-seeded agretti may protect itself from excessive B in the soil and irrigation water by limiting its B uptake. Reducing B accumulation can be a plant defense-like response to excessive B in the root zone is imperative for any crop considered for growing in soil or with irrigation water containing B. Boron in irrigation water is toxic to typical agronomic crops, as described in Reid et al. (2004), Reid and Fitzpatrick (2009), at concentrations greater than 4 mg B/L.

Developing a Se-biofortification strategy with a saline water reuse system in the westside of SJV in Central California, importantly requires the identification of a cropping system with high salt and B tolerance and selecting a crop that has economic value for the growers. In this study, we have shown that producing Se-enriched agretti under organic- like growing conditions successfully produces a viable crop.

It is important to note the presence of high levels of Na in the shoots (5 to 9.8% DW) when growing Se-biofortified agretti under these tested saline growing conditions. Consequently, consumption of agretti produced under saline

growing conditions should be monitored for people requiring a low Na diet. Further studies may explore the double potential of dried agretti biomass as Se-enriched food and as organic salt replacement, thus increasing the economic value of the plant.

Currently, studies on consumers' acceptance and market viability of Se-enriched agretti are being carried out in Central California (Lone, unpublished). Preliminary results indicate that consumers will need more education about Se-enriched halophyte plants and the safety on using saline irrigation on food crops. Others have reported on the importance of having consumer acceptance of Se-enriched food products (Cox and Bastiaans, 2007; Wortmann et al., 2018). This acceptance is applicable for Se-biofortified agretti produced from saline growing conditions since a typical consumer is not aware of saline irrigation practices.

Most of the risks associated with the reuse of Se-enriched saline-sodic waters are related to degrading soil quality and to paying close attention to Se content in edible plant tissue (Imoff, 1991; Oster and Grattan, 2002; Grattan et al., 2014). Moreover, levels of other trace elements naturally present in westside soils of the SJV should also be closely monitored (Grattan and Oster, 2003; Suyama et al., 2007). To reduce the impact on the environment and human and livestock health, the sustained use of saline water for biofortifying crops with Se requires the implementation of special management practices, such as the biological management of salts, i.e., Na, by selecting salt tolerant agretti as a companion crop for Na removal (Colla et al., 2006) and the adoption of irrigation management practices when using saline water.

Conclusion

This study identified a Se-biofortification strategy with the production of agretti using saline, B- and Se-laden soil and irrigating with saline and low-saline water, respectively. This is one of the first investigations on growing agretti as a Se-biofortified crop under organic-like field agriculture practices and irrigating with different amounts of low-saline and saline water in the west side of the SJV in California. To our knowledge, there is no information available on both producing Se-biofortified agretti or on production with irrigation of low-saline or saline water under high saline and B growing conditions.

There is a potential for the producing of Se-enriched agretti at in the saline soils of the SJV and similar arid areas with similar geological sources of Se. Because the sustainability of producing typical agronomic crops in California is decreasing due to a lack of good quality water, alternative salt and B tolerant crops need to be identified to accumulate Se, despite both excessive salts in soil and irrigation water. It is important that selected Se-biofortified crops like agretti have economic value and have farmers who will accept growing a new crop. In this regard, previous studies have shown a positive response to the marketability and consumption of agretti by the retail industry, gastronomy, and consumers (Lone, unpublished). The feasibility of large-scale production of Se-enriched, agretti depends on improving agronomic practices such as improved seed viability, germination potential, and optimal growth conditions. Importantly, consumer acceptance for consuming new Se-enriched crops produced from saline waters must also be take into consideration. For example, surveys conducted by Lone et al. (unpublished) indicate approximately three quarters of respondents have 'no knowledge' of halophyte plants such as agretti and 77.4% are not aware halophytes are food. When shown photos of agretti, only a small proportion of respondents know about the crop, but 92.7% are willing to try it, and 76.7% want it offered where they purchase food. When queried about irrigation water, 55.6% had 'some knowledge' about drainage and poor quality water irrigation, but only 13.7% were aware that saline drainage water can be used for irrigation of food crops. This general lack of knowledge about growing conditions may stem from consumers not fully understanding terms such as 'saline,' 'non-saline,' and 'drainage water' that were defined and used in the survey. Thus, marketers should be cognizant that additional consumer education and use of 'consumer friendly' terminology may be necessary when introducing new Se-enriched food products in the marketplace.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

GB: conceptualization, methodology, reviewing, and editing. TC: writing – original draft preparation, methodology, and data analysis. MZ: assisting with data curation. KV and TL: reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.996502/full#supplementary-material

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Selenium supplementation and pregnancy outcomes

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In vertebrates and invertebrates, selenium (Se) is an essential micronutrient, and Se deficiency or excess is associated with gonadal insufficiency and gamete dysfunction in both males and females, leading to implantation failure, altered embryonic development and, ultimately, infertility. During pregnancy, Se excess or deficiency is associated with miscarriage, pre-eclampsia (hypertension of pregnancy), gestational diabetes, fetal growth restriction and preterm birth. None of this is surprising, as Se is present in high concentrations in the ovary and testes, and work in animal models has shown that addition of Se to culture media improves embryo development and survival *in vitro* in association with reduced reactive oxygen species and less DNA damage. Selenium also affects uterine function and conceptus growth and gene expression, again in association with its antioxidant properties. Similarly, Se improves testicular function including sperm count, morphology and motility, and fertility. In animal models, supplementation of Se in the maternal diet during early pregnancy improves fetal substrate supply and alters fetal somatic and organ growth. Supplementation of Se throughout pregnancy in cows and sheep that are receiving an inadequate or excess dietary intake affected maternal whole-body and organ growth and vascular development, and also affected expression of angiogenic factors in maternal and fetal organs. Supplemental Se throughout pregnancy also affected placental growth, which may partly explain its effects on fetal growth and development, and also affected mammary gland development, colostrum yield and composition as well as postnatal development of the offspring. In conclusion, Se supplementation in nutritionally compromised pregnancies can potentially improve fertility and pregnancy outcomes, and thereby improve postnatal growth and development. Future research efforts should examine in more detail and more species the potential benefits of Se supplementation to reproductive processes in mammals.

KEYWORDS

selenium, supplementation, pregnancy, ovary, testis, fetus, offspring, developmental programming

Introduction

In both vertebrates and invertebrates, selenium (Se) deficiency or excess is associated with infertility (that is, the inability to conceive and establish a pregnancy), as reflected by small, poorly developed and poorly functioning gonads, primarily ovarian follicles in females and testes and spermatozoa in males. At least a portion of this problem is associated with implantation failure due to poor embryonic development and altered endometrial (uterine) function. Deficiency or excess of Se also is associated with reduced libido. Lastly, in terms of pregnancy, Se excess or deficiency is associated with spontaneous abortion (miscarriage), pre-eclampsia (hypertension of pregnancy), gestational diabetes, fetal growth restriction, and preterm birth (1–4).

Consistent with its known functions in cellular metabolism, in reproductive tissues Se appears to function primarily as a component of selenoproteins/selenoenzymes in a variety of antioxidant systems, including glutathione peroxidases (GPX), iodothyronine deiodinases (DIO), and thioredoxin reductases (TXNR). These major familes of antioxidant enzymes contribute to reductions in tissue reactive oxygen species and therefore minimize DNA damage (5).

Selenium in the female reproductive tract

The bovine ovary contains high levels of Se (**Figure 1**), which are localized to healthy preovulatory follicles, but not in atretic follicles (6). Localization of Se in healthy pre-antral follicles indicates that Se is in close contact with the pre-ovulatory oocyte, which may play a preparatory role for subsequent fertilization, embryo development, and postnatal life. In humans, cases of low plasma, follicular fluid, amniotic fluid or tissue Se concentrations and(or) low tissue GPX concentrations or activity are associated with unexplained infertility, miscarriage, preterm birth, gestational diabetes mellitus, and small for gestational age (SGA) fetuses/newborns (1, 2, 7–9). Elevated serum levels of Se-binding protein 1, an autoantibody produced by the ovary, has been reported in women with unexplained intertility and premature ovarian failure (1, 10). In women with gestational diabetes mellitus, serum Se levels were low, and Se supplementation improved glycemic status and lipid profiles (11, 12).

After ovulation the oocyte moves to the oviduct, where fertilization and early embryo development take place. Oviductal fluid is secreted by the oviduct and acts as an embryotropic culture media for the oocyte and early embryo for their time in residence (13, 14). Addition of Se to *in vitro* fertilization cultures in animal models (cattle, dogs, pigs, yak, etc.) has resulted in positive impacts on embryo development and survival, reduced reactive oxygen species, and reduced DNA damage (15–19). Interestingly, Se-dependent mechanisms are in place to control embryo metabolic reprogramming in pro-inflammatory environments (20).

Upon deposition of semen into the reproductive tract a post-mating inflammatory response is elicited (21), and an LPS challenge of cultured bovine endometrial cells demonstrated a protective role of Se (22). *In vivo* effects of Se were demonstrated in cattle, where females receiving an organically bound source of Se had greater conceptus length compared with females receiving an inorganic source of Se (23). In addition, cattle receiving organic Se had differential expression of genes related to maternal recognition of pregnancy, including interferon-stimulated genes and progesterone-stimulated genes (23).

Selenium in the male reproductive tract

The testis contains high concentrations of Se (**Figure 2**), where Se has effects both in the seminiferous tubule where sperm are being produced, and in the interstitial space where testosterone production occurs and the blood supply resides (4). As sperm mature Se is localized in the mid-piece, which is also the location of sperm mitochondria (24). The action of Se is primarily as GPX4, which protects sperm from oxidative damage to their cell membranes and DNA. However, there also appears to be a specialized testes- specific isoform of TXNR (5, 25), which supports the importance of Se-containing antioxidant enzymes to testicular function and health.

In addition, greater dietary intake of Se has been associated greater sperm concentrations in semen of men infertile men (26) and some Se supplementation studies in infertile men show improvements in testicular antioxidant activity, semen Se concentrations, sperm count, sperm morphology and motility, and fertility (1–3). Selenoproteins are abundant in the testis and epididymis, include GPX4 (testis, intracellular membranes), sperm nucleus GPX4 (snGPX4), mitochondrial GPX4 (mGPX4; sperm midpiece – see **Figure 2**), cytosolic GPX4 (cGPX4; testis and epididymal epithelium), secreted GPX5 (epididymal lumen), cytosolic GPX3 and GPX1 (epididymal epithelium) (3). In addition, gene knockouts of selenoproteins in male mice, including mGPX4, SELENOP, snGPX4, GPX5 and global GPX4 (mGPX4, snGPX4, and cGPX4), lead to sperm abnormalities, defects in chromatin condensation in sperm, early embryonic death, and(or) increased number of miscarriages, developmental defects and neonatal mortality (3).

In terms of our understanding of the underlying mechanisms, Se excess or deficiency affects the concentrations or activities of various selenoproteins, resulting in:

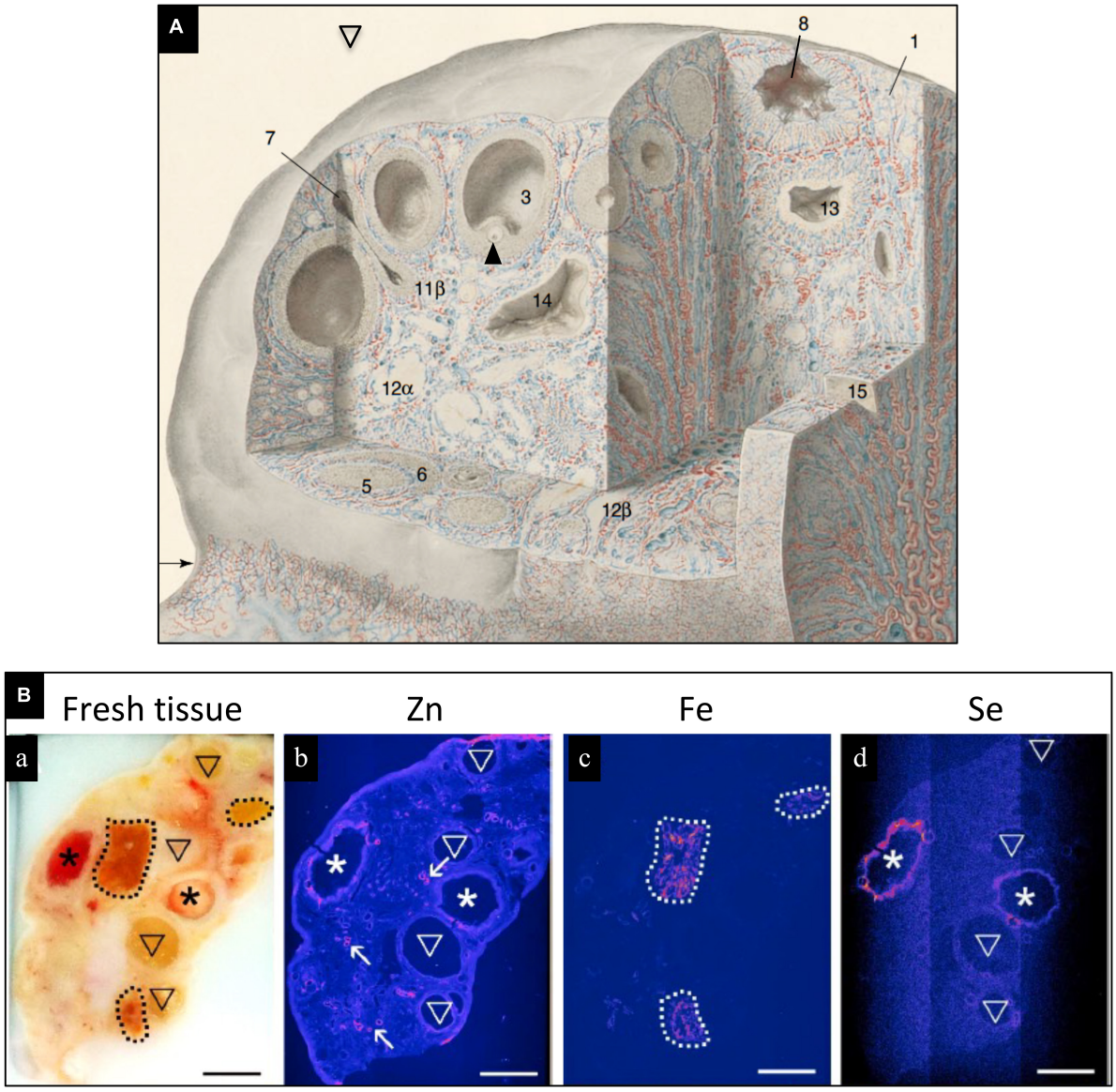

FIGURE 1
(A) Drawing of human ovary with sections removed to reveal histological details of an antral (preovulatory) follicle (3) containing an oocyte (arrowhead) and a postovulatory follicle that has released its oocyte and partially collapsed (8). Blood vessels are colored red (arteries) and blue (veins); modified, with permission, from Clark, 1900. **(B)** Trace elements localized in bovine ovaries by synchrotron x-ray fluorescence (S-XRF). (a) Represents fresh tissue. Zinc (b, pink) localized primarily to blood vessels, Fe (c, pink) localized primarily to corpora lutea, and Se (d, pink) localized to healthy, preovulatory follicles (*) but not to atretic (regressing) antral follicles. Modified, with permission, from Ceko et al. (6).

- Oxidative stress/DNA damage from reactive oxygen species;
- Lack of structural integrity of sperm, affecting sperm motility and fertilization capacity;
- Defects in transport of Se into tissues, particularly testis and brain;
- Alterations in other Se effects/functions – e.g., altered gonadal morphology/size, endocrine function (e.g., thyroid), immune function, cardiovascular function, synergism with Vit E, etc. (27).

Selenium supplementation during pregnancy

There are geographic locations and times of the year when forages grazed by livestock have insufficient Se to meet requirements. In addition, producer decisions about whether to provide supplemental mineral to grazing livestock vary widely. Therefore, our research group implemented a bovine model comparing unsupplemented beef heifers to those receiving a

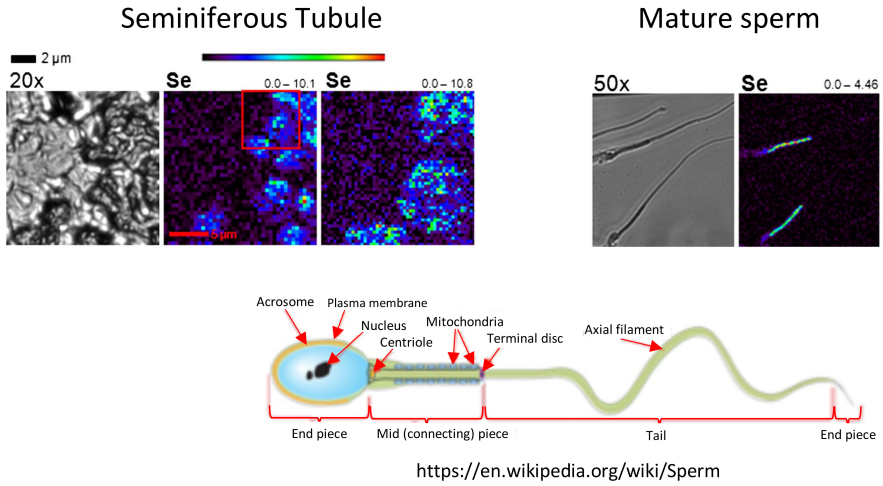

FIGURE 2
Localization of Se in mouse testis by x-ray fluorescence microscopy (XFM). In the left-hand images, a seminiferous tubule is visualized in cross section at 20×. Se (bright blue to green) localizes to elongating spermatids. In the right-hand images, Se in mature sperm localizes to the mid-piece, which is the location of sperm mitochondria (see the schematic of a mature sperm, lower right). The range below the seminiferous tubule image indicates the low and high concentrations of Se in ng/cm2. Modified, with permission, from Kehr et al. (24).

Se-containing mineral supplement (VTM) to understand the impacts of early gestation supplementation on maternal and fetal outcomes (28–33). An important aspect of these studies is that the control, unsupplemented heifers were receiving a basal diet that was either inadequate or in excess of requirements, both of which typically result in reduced birth weights of the offspring. Thus, they were also receiving inadequate or excess micronutrient intakes.

Evaluation of maternal and fetal samples collected on d 83 of gestation revealed heavier livers in fetuses exposed to VTM during gestation (29), and that concentrations of Se in maternal liver, fetal liver, muscle, and allantoic fluid were all greater in heifers receiving the VTM supplement. In addition, concentrations of Se in maternal liver were correlated with concentrations in fetal liver (r = 0.60), fetal muscle (r = 0.40), and allantoic fluid [r = 0.34; (33)]. Though no differential expression of selenoprotein transcripts was observed in the fetal or maternal portions of the placenta, VTM supplementation influenced genes related to amino acid activation, fat cell differentiation and metabolic processes (32). Amino acids are critical fuels for fetal growth and development (34) and our evaluation revealed that total amino acids and concentrations of 12 of 14 neutral amino acids evaluated in allantoic fluid were greater in heifers receiving VTM (28). Taken together, our results demonstrate that providing a Se-containing supplement during early gestation resulted in major alterations in substrate supply and(or) utilization in the fetus, indicating that research evaluating post-natal effects on health, growth, and metabolism is necessary.

In a series of studies we targeted feeding "supranutritional" (meaning above adequate but below toxic) levels of Se to pregnant ewes, fed as Se-enriched yeast or Se-enriched wheat (35–48). Again, the control, unsupplemented animals were receiving a basal diet that was either inadequate or in excess of requirements, both of which typically result in reduced birth weights of the offspring.

When fed during early pregnancy (from 21 days before until 64 days after breeding – i.e., 0.44 of pregnancy), supranutritional Se increased maternal lung mass, liver mass, and total visceral organ mass, as well as cellularity, cell proliferation and vascularity of maternal small intestine. All of these effects on the maternal system would increase metabolic capacity to support the metabolic demands of pregnancy.

Supranutritional levels of Se in the maternal diet during early pregnancy also increased fetal body mass, heart mass, lung mass, spleen mass, total visceral organ mass and large intestinal mass, as well as cell density of fetal skeletal muscle. These effects of Se supplementation would potentially improve survival and growth of the fetus and offspring. In addition, the effect on fetal skeletal muscle also has important implications for postnatal growth and carcass quality, considering that the number of myocytes in skeletal muscle is "fixed" at birth (49).

When fed throughout pregnancy, supranutritional levels of Se in the maternal diet also affected maternal whole-body and organ growth and vascular development, and these effects depended on the plane of nutrition (adequate or restricted intake). For example, Se supplementation increased maternal mammary gland vascularity at 24 h postpartum, Selenium supplementation also increased fetal body weight as well as fetal heart, lung, spleen, total visceral and large intestine weights and fetal muscle DNA concentations at 0.9 of gestation. Along with the effects on vascular development, supplemental Se

throughout pregnancy also increased maternal and fetal organ expression of mRNA for vascular growth (angiogenic) factors, including NOS3 and VEGF.

Supranutritional Se fed to ewes throughout gestation also increased cell density and cell proliferation in the placenta in late pregnancy as well as lamb birth weights. As the placenta is the only source of exchange of nutrients, respiratory gases and metabolic wastes between the fetal and maternal systems (50–52), the effects of Se on placental development may explain, at least in part, the effects of supranutritional Se on fetal growth and development.

Alternatively, epigenetic mechanisms within developing offspring may also explain developmental programming responses resulting from dietary Se supplementation (53). Specifically, enzymes associated with one-carbon metabolism have been shown to be affected by Se (54, 55), while others (56) reported that Se regulates microRNAs (56) and DNA methylation (55, 57). In humans experiencing Kashin-Beck disease (associated with Se deficiencies), differentially methylated genes were reported (53). Research exploring the potential role of Se induced epigenetic changes in offspring within a developmental programming paradigm are needed to further understand the mechanisms and roles of supplemental dietary Se in developmental programming events in livestock.

Lastly, supranutritional Se fed to ewes throughout gestation also increased colostrum yield, altered colostrum composition, and increased mammary gland vascular development, and resulted in increased average daily weight gain, efficiency of growth, visceral adiposity and small intestinal mass and vascular development of the lambs postnatally. These observations further suggest a role for supranutritional supplementation of Se to the dams on developmental programming of the offspring and support the need for additional research in this area.

Conclusion

As we have discussed, Se plays an important role in reproductive processes. Recent research with Se supplementation of sheep during nutritionally compromised pregnancies has suggested that "supranutritional" levels in the diet can positively impact pregnancy outcomes. However, these studies need to be replicated in other mammals as well. In addition, the effects of Se supplementation on other reproductive processes such as follicular development, oocyte and sperm development and maturation, fertilization and implantation, early embryonic development, and, especially, developmental programming of offspring, warrant further research as well (4, 58).

Importantly, when supranutritional maternal Se was fed as sodium selenate at 20 or 100×, or as Se-enriched wheat at 20×, of so-called "adequate" levels from day 50 to 134 (0.34–0.92) of pregnancy in ewes, no signs of selenosis were observed. These studies using sheep models of pregnancy therefore indicate that in addition to the role of dietary Se in other reproductive processes, supranutritional levels of Se fed to ewes during the periconceptual period or throughout pregnancy are not only non-toxic but can improve maternal and fetal pregnancy outcomes and postnatal growth and development. Taken together, these observations suggest to us that further research on adding Se to the diet during pregnancy is warranted in other mammals as well.

Author contributions

All authors contributed equally to the preparation of this manuscript.

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Conflict of interest

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

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Separate foliar sodium selenate and zinc oxide application enhances Se but not Zn accumulation in pea (*Pisum sativum* L.) seeds

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Up to 15% and 17% of the world population is selenium (Se) and zinc (Zn) deficient, respectively. Pea (*Pisum sativum* L.) is an important staple legume with a high potential for Se and Zn biofortification in seeds. A 2-year pot experiment investigated two pea varieties (Ambassador and Premium) following foliar-applied sodium selenate (0/50/100 g of Se/ha) and zinc oxide (0/375/750 g of Zn/ha) at the flowering stage. Selenate and zinc oxide had minimal overall effects on growth parameters. Zinc oxide did not improve Zn accumulation in both seed varieties, while selenate improved Se accumulation in both seed varieties dose-dependently. Premium accumulated greater amounts of Se in seeds than Ambassador ($p < 0.001$). Selenium concentrations were highest in seeds of Premium treated with 100 g of Se/ha [7.84 mg/kg DW vs. the control (0.16 mg/kg DW), $p < 0.001$]. The predominant Se species in Se-enriched seeds was selenomethionine (40%–76% of total Se). Furthermore, a significant ($p < 0.01$) positive correlation was found between Zn and S concentrations in Ambassador ($r^2 = 0.446$) and Premium ($r^2 = 0.498$) seeds, but not between Se and S. Consuming as little as 55 g/day of pea biofortified by 50 g of Se/ha would cover 100% of the adult RDA (55 µg) for Se. Findings are important for improving foliar biofortification of pea with Se and Zn.

KEYWORDS

legume biofortification, selenate, zinc oxide, mineral deficiency, nutrition, food security, HPLC-ICP/MS

Introduction

Selenium (Se) and zinc (Zn) are essential trace elements for humans. Selenium (as selenoproteins) and Zn are involved, *via* their role as enzymatic co-factors, in a large number of antioxidant defense and immune functions, such as being co-factors for glutathione peroxidase and superoxide dismutase, respectively (Marreiro et al., 2017; Barchielli et al., 2022). A limited intake or low circulating concentrations of Se and Zn have been associated with increased risk of mortality and several non-communicable chronic diseases. These include cancer, neurodegenerative diseases, and cardio-metabolic complications, such as type 2 diabetes or metabolic syndrome (Rayman, 2012; Kaur et al., 2014; Barchielli et al., 2022). A relative lack of Se and Zn has also been associated with infectious diseases, including COVID-19, likely due to their participation in antioxidant and anti-inflammatory and thus immune-relevant processes in the body (Alexander et al., 2020; Du Laing et al., 2021). However, excessive intakes of both Se and Zn may also cause health problems, as reviewed previously (Rayman, 2020; Agnew & Slesinger, 2022).

According to some sources, it is estimated that up to 15% and 17% of the world population is Se and Zn deficient, respectively (Kumssa et al., 2015; Shreenath et al., 2022). Suboptimal Se and Zn statuses were reported to be also widespread throughout Europe. This reflects, to a large extent, inadequate soil levels (Tóth et al., 2016), as they have become depleted by agricultural use and rainfall. Main dietary sources for Se include cereals and grains, while for Zn, meat and meat products are the prominent source (Olza et al., 2017), though cereals and grains are the second predominant source. Therefore, intakes of Se and Zn depend largely on their concentrations in soil and the bioavailability from major crops (Alloway, 2008; Chilimba et al., 2011; Winkel et al., 2015). However, soil Se and Zn are uneven in their distribution and availability to plants, as reviewed earlier (Sadeghzadeh, 2013; Jones et al., 2017).

Biofortification is a promising agricultural strategy to improve the level of micronutrients in staple foods. This strategy encompasses classical plant breeding, genetic engineering, and agronomic biofortification. The latter is based on optimized fertilizer application to the soil and/or crop leaves in the case of foliar biofortification, as reviewed previously (de Valença et al., 2017; Cakmak and Kutman, 2018; Szerement et al., 2022). It has been shown that foliar spraying is a highly effective method of plant biofortification for Se and Zn (Delaqua et al., 2021; Sattar et al., 2021). The efficiency of foliar applied trace elements is affected by numerous factors. These include physicochemical properties of the formulation, the environmental conditions under which spraying is carried out, or the characteristics of the plant to which spraying is applied, as reviewed previously (Fernández and Brown, 2013).

Legumes constitute staple foods for billions of people around the world. However, legume biofortification has been emphasized as an underexploited strategy for combatting hidden hunger (Rehman et al., 2018; Kumar and Pandey, 2020). Pea (*Pisum sativum* L.) is an important legume crop produced worldwide and employed for animal and human nutrition. In 2020, the world production of dry peas amounted to 14.6 million tons, with cultivated areas covering 7.2 million hectares (FAOSTAT 2022). Pulses (including peas) are beneficial for sustainable agriculture and environment, biodiversity, global health, and food security (Powers & Thavarajah, 2019; Ferreira et al., 2021). These crops are of high nutritional value, play an essential role in cropping systems, enhance soil health, and reduce synthetic nitrogen fertilizer applications and associated fossil energy consumption (Sahruzaini et al., 2020; Ferreira et al., 2021). Peas are a good and affordable source of high-biological-value protein (Ge et al., 2020), complex carbohydrates, dietary fiber, starch, vitamins, minerals, and phytochemicals that may favorably affect human health (Dahl et al., 2012). The intake of peas and their constituents has been associated with metabolic, cardiovascular, and gastrointestinal health benefits (Dahl et al., 2012; Kumari and Deka, 2021). Regarding Se, its availability from crops does, to a large degree, depend not only on the total amount of Se but also on the chemical speciation of Se in the food crops. It is understood that organic Se species are absorbed more effectively and are considered less toxic at higher intakes than inorganic species (Zhang et al., 2013). However, studies on Se speciation in legumes are also limited (Smrkolj et al., 2006; Poblaciones et al., 2014; Thavarajah et al., 2015).

The aim of this 2-year pot experiment was to examine the effect of foliar-applied Se (sodium selenate) and Zn (zinc oxide) at the flowering stage on two pea varieties (Ambassador and Premium). The experiment consisted of five treatments, including one un-amended control and two levels of applications for both Se and Zn. Growth parameters; Se, Zn, and sulfur (S) concentrations (due to potential interactions with Se); and Se speciation were determined in seeds. To the best of our knowledge, the present investigation is only the second study to investigate Se speciation following foliar biofortification of peas (Smrkolj et al., 2006).

Materials and methods

Chemicals

Zinkuran SC was purchased from Arysta LifeScience Slovakia s.r.o. (Nové Zámky, Slovakia). Sodium selenate was obtained from Alfa Aesar (Karlsruhe, Germany). Nitric acid (HNO_3, for trace element analysis) and hydrogen peroxide 30% (Suprapur) were acquired from LGC Standards (Molsheim, France) and Merck/VWR (Leuven, Belgium), respectively. Sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), Se-

methionine (SeMet), Se-cystine ($SeCys_2$), and Se-methyl-selenocysteine (SeMetSeCys) were purchased from Sigma Aldrich (St. Louis, MO, USA). Protease XIV, citric acid, and methanol were from Sigma Aldrich. MilliQ (MQ) water from Water Systems Ltd. (Brussels, Belgium) was used throughout the experiment.

Design of experiment and sample preparation

A 2-year outdoor pot experiment, during which plants were not fully exposed to outdoor conditions, was conducted in 2014 and 2015 in the Botanical Garden of the Slovak University of Agriculture in Nitra (48.305 N, 18.096 E), Slovakia. The experiment was arranged with four replicates per treatment, two pea varieties, and five different treatments (total of 80 pots over two growing seasons). The average monthly air temperature and total monthly rainfall in the 2014 growing season were as follows: March (9.3°C and 15.4 mm), April (12.4°C and 48.9 mm), May (15.2°C and 57.6 mm), and June (19.3°C and 52.5 mm), while in the 2015 growing season, the corresponding values were as follows: March (6.3°C and 35.4 mm), April (10.4°C and 25.0 mm), May (15.1°C and 69.5 mm), and June (19.9°C and 10.2 mm). A gleyic fluvisol soil (that is the typical soil type of the area) was employed in the experiment. The soil from the 2014 growing season had a pH of 6.47 and contained 19.5 mg kg^{-1} of N, 86.3 mg kg^{-1} of P, 498 mg kg^{-1} of K, 6,610 mg kg^{-1} of Ca, 816 mg kg^{-1} of Mg, 26.3 mg kg^{-1} of S, 2.47 mg kg^{-1} of Zn, 0.08 mg kg^{-1} of Se, and 3.46% of humus. The soil from the 2015 growing season had a pH of 7.16 and contained 19.1 mg kg^{-1} of N, 245 mg kg^{-1} of P, 150 mg kg^{-1} of K, 6,340 mg kg^{-1} of Ca, 644 mg kg^{-1} of Mg, 7.5 mg kg^{-1} of S, 2.39 mg kg^{-1} of Zn, 0.08 mg kg^{-1} of Se, and 3.25% of humus. Soil was collected with a soil corer with a sampling depth of 0–0.3 m. The concentration of elements in soils was determined according to the method of Varényiová et al. (2017) for total N and S, and available P, K, Mg, and Ca; Ducsay et al. (2009) for total Se; and Lindsay and Norvell (1978) for available Zn.

Two pea varieties, i.e., Ambassador (late variety, restored hybrid) and Premium (early variety, open pollinated), were selected for the experiments. Seeds were purchased from a local farmer. Ten-liter plastic square pots were filled with soil and placed in a wire mesh housing to protect plants against bird attacks. Thirty seeds/pot were sown in two rows at 5 cm depth in mid-March. Selenium as sodium selenate and Zn as Zinkuran SC (30% ZnO + 6% chelate) were applied in the experiment. The experiment consisted of five treatments: un-amended control (control), 50 g of Se/ha (Se1), 100 g of Se/ha (Se2), 375 g of Zn/ha (Zn1), and 750 g of Zn/ha (Zn2). The solutions employed contained 0.1 and 0.2 g/L of Se and 0.75 and 1.5 g/L of Zn. Foliar applications of Se and Zn were performed at the flowering stage of plants during non-rainy periods. A plastic trigger spray bottle was used for the manual application of fertilizers. No additional fertilization was employed. Watering and weed and snail removal were carried out regularly. Toxic effects of foliar Se and Zn treatments on plants or incidences of pests and diseases were not observed during the experiment. Freshly harvested seeds were immediately lyophilized, homogenized by grinding, and the concentrations of Se, Zn, and S, and Se species were examined.

Growth parameters

Number of seeds per pod, pod length, and pod perimeter were measured after harvest. Samples were dried at 105°C in a drying oven to a constant weight for seed dry matter determination.

Concentrations of total Se, Zn and S in seeds

An aliquot (0.2 g) of each sample was mixed with 3.5 ml of HNO_3 (65%) and 3.5 ml of H_2O_2 (30%). Thereafter, microwave digestion for complete combustion of organic matrix was carried out using a MARS 6 system (CEM, Orsay Cedex, France, 1,200 W, 10 min at 55°C, 10 min at 75°C, and 45 min at 120°C). Total Se, Zn, and S concentrations [mg/kg dry weight (DW)] were subsequently determined in the diluted digests *via* an inductively coupled plasma mass spectrometer (ICP-MS, PerkinElmer Elan DRCe, Waltham, MA, USA, for Se) and an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian Vista MPX, Palo Alto, CA, USA, for Zn and S), respectively. External calibration was used. Accuracy and precision were monitored by periodic evaluation of a calibration blank, re-analyzing standards during sample runs, and analysis of certified reference materials (including rice flour NIST1568a, sea lettuce BCR279 and spinach leaves SRM 1570a), spiked samples and analytical duplicates. Analytical batches were rejected and reanalysis was planned when the concentrations measured in reanalyzed standards, certified reference materials, or spiked samples deviated more than 10% from the expected/certified value.

Selenium speciation in seeds

Selenium speciation analysis was determined according to Lavu et al. (2013); Lavu et al. (2012). The seeds of pea (Ambassador and Premium variety) treated with 100 g of Se/ha as selenate were selected for Se speciation analysis. Specifically, 0.2 g of whole plant samples and 80 mg of the enzyme protease XIV were dispersed in 5 ml of water in a 10-ml centrifuge tube. The mixture was shaken for 24 h at 37°C and centrifuged for 30 min at 10,000 *g*. The supernatant was filtered through a 0.25-μm syringe PVDF membrane filter. The filtrate was analyzed for Se speciation by an ICP-MS (PerkinElmer DRC-e, Sunnyvale, CA, USA) coupled to a high-performance

liquid chromatograph (Series 200 HPLC, Perkin Elmer, Sunnyvale, CA, USA), respectively. A Hamilton PRP-X100 anion exchange column (250 mm × 4.6 mm, 5 µm) was used as stationary phase in the HPLC instrument. The isocratic mobile phase was 10 mM citric acid with 5% (v/v) methanol, adjusted to pH 5.0. The standard solutions of the different Se species were prepared with sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), Se-methionine (SeMet), Se-cystine ($SeCys_2$), and Se-methyl-selenocysteine (SeMetSeCys).

Statistical analysis

Normal distribution of data and equality of variance were verified by normality plots and box plots, respectively. Whenever required, data were log-transformed in order to achieve normal distribution. Multivariate models were then employed, with seed dry matter, number of seeds per pod, pod perimeter, pod length, and Se, Zn, and S concentrations in seeds as the observed (dependent) variables, and genetic variant (two levels), year (two levels), and biofortificant type (five levels, two for Se, two for Zn, and controls) as independent, fixed factors. Biofortification levels were nested within biofortificant. Following significant Fisher *F*-tests, all group-wise comparisons were carried out (Bonferroni post-hoc tests). In case of significant interactions, models were re-run with one of the significant interacting terms kept constant. A p-value <0.05 (two-sided) was considered statistically significant. SPSS, version 25.0 (IBM, Chicago, IL, USA), was used for all analyses including Pearson correlation analyses.

Results

Growth parameters

Following multivariate models, combined analysis of variance showed that treatment (pooled years and varieties) significantly affected all examined variables except for the number of seeds per pod. Growing year (pooled treatments and varieties) had a significant effect on all variables except for pod length. Variety (pooled treatments and years) showed a significant effect on all variables. Interactions were significant in some cases (Table 1).

When investigating effects per year, in 2014, the Zn1 treatment significantly decreased seed dry matter of Ambassador vs. the control, while Se1, Se2, and Zn2 significantly decreased seed dry matter of Premium vs. the control. Ambassador showed significantly higher seed dry matter than Premium for the control, Se1, Zn2, and a trend for Se2 treatment. Also, Zn1 and Zn2 significantly increased the number of seeds per pod of Ambassador vs. the control. In contrast, Zn1 and Zn2 significantly decreased the number of seeds per pod of Premium vs. the control. Premium showed a significantly higher number of seeds per pod than Ambassador for Se2. Ambassador showed a significantly higher number of seeds per pod than Premium for Zn1 and Zn2. In 2014, treatment did not significantly influence pod length of Ambassador vs. the control, while it had a marginal significant effect on the pod length of Premium vs. the control (though individual group-wise comparison with post-hoc correction did not reveal differences due to correction for multiple comparison). Ambassador showed a significantly higher pod length than Premium for all treatments. Finally, in 2014, treatment did not significantly affect pod perimeter of Ambassador vs. the control. Pod perimeter of Premium was significantly increased by Se1 and Se2 vs. Zn1 and Zn2. Premium showed significantly higher pod perimeter than Ambassador for Se2.

In 2015, the Zn1 treatment significantly decreased seed dry matter of Ambassador vs. the control, while the Se2 treatment significantly increased seed dry matter of Premium vs. the Zn2 treatment. Ambassador showed significantly higher seed dry matter than Premium for the control, Se1, and Zn2. Furthermore, for 2015, treatment did not significantly affect the number of seeds per pod of both varieties vs. controls. No

TABLE 1 Combined analysis of variance for the effects of year, variety, and treatment on seed dry matter, number of seeds per pod, pod length, pod perimeter, and seed Se, Zn, and S concentrations.

	DF	Seed dry matter (%)	Number of seeds/pod	Pod length (cm)	Pod perimeter (cm)	Se (mg/kg DW)	Zn (mg/kg DW)	S (mg/kg DW)
Year (Y)	1	<0.001	<0.001	NS	<0.001	NS	<0.001	0.002
Variety (V)	1	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	NS
Treatment (T)	4	<0.001	NS	0.049	0.025	<0.001	NS	NS
Y × V	1	0.001	<0.001	<0.001	0.044	0.020	0.001	0.008
Y × T	4	0.007	NS	NS	NS	0.026	0.011	NS
V × T	4	<0.001	<0.001	NS	0.012	NS	0.002	NS
Y × V × T	4	0.003	<0.001	NS	0.019	NS	NS	NS

DF, degrees of freedom; NS, not significant.

significant differences were found for the number of seeds per pod between Ambassador and Premium for all treatments. Treatment did not significantly affect pod length of both varieties vs. controls. Ambassador showed significantly higher pod length than Premium for Se2 and Zn1. Finally, in 2015, treatment did not significantly influence pod perimeter of both varieties vs. controls. Ambassador showed significantly higher pod perimeter than Premium for Se1, Se2, Zn1, and Zn2.

Comparing growing year per variety, it significantly affected seed dry matter and the number of seeds per pod of both varieties, it had a significant effect on the pod perimeter of Premium (not Ambassador), though it did not significantly affect pod length of both varieties (Table 2).

Se, Zn and S concentrations in seeds

Following multivariate models, combined analysis of variance showed that treatment (pooled years and varieties) significantly affected only Se concentration. Growing year (pooled treatments and varieties) had a significant effect on Zn and S concentrations. Variety (pooled treatments and years) showed a significant effect on Se and Zn concentrations. Interactions were significant in some cases (Table 1).

For both years, Se treatment significantly increased Se concentration vs. controls in Ambassador and Premium. In 2014, the highest Se concentration was found in Premium treated with Se2 vs. the control and in Ambassador treated with Se2 vs. the control. Also, no significant differences were observed in Se concentration between Ambassador and Premium for Se1 and Se2. Premium showed significantly higher Se concentration than Ambassador for the control. In 2015, the highest Se concentration was found in Premium treated with Se2 and Se1 vs. the control. Also, Premium showed significantly higher Se concentration than Ambassador for Se1 and Se2 in 2015. Contrarily, Ambassador showed slightly but significantly higher Se concentration than Premium for the control (Figure 1A). Growing year had no significant effect on Se concentration in both varieties ($p > 0.05$).

In 2014, treatment did not significantly influence Zn concentration vs. controls in both varieties. Ambassador showed significantly higher Zn concentration than Premium for all treatments. Also, treatment did not significantly affect S concentration vs. controls in both varieties. No significant differences were observed in S concentration between Ambassador and Premium for all treatments. In 2015, the Zn1 treatment significantly decreased Zn concentration vs. the control in Ambassador, while Se1 significantly decreased Zn concentration vs. the control in Premium. Ambassador showed significantly higher Zn concentration than Premium for the control, Se1, Se2, and a trend for Zn2 treatment (Figure 1B). Also, treatment significantly influenced S concentration vs. controls in both varieties. In Ambassador, Se1 and Zn2 decreased S concentration vs. the control, while in Premium, Zn1 increased S concentration vs. the control. Premium showed significantly higher S concentration than Ambassador for all treatments (Figure 1C). Growing year significantly affected Zn concentration in both varieties ($p < 0.001$), and it had a significant effect on S concentration in Premium ($p = 0.005$) compared with Ambassador ($p = 0.075$).

Se speciation in seeds

Selenium species recovery ranged between 62% and 106% after protease hydrolysis for samples treated with 100 g of Se/ha (Table 3). The chromatogram of the standard solution containing all determined Se species is shown in Figure S1C. The predominant Se species identified in pea seeds was SeMet, ranging between 58% and 76% of total Se in Ambassador and between 40% and 71% of total Se in Premium. The species SeCys, SeMetSeCys, Na_2SeO_3, and Na_2SeO_4 were also identified, although in much lower proportions (Table 3 and Figure S1).

Correlations

Significant, strong, and positive correlations between Se dose and seed Se concentration were found for Ambassador from 2014 and for Premium from 2014 and 2015, all with an r^2 above 0.98 (Table S1). For Ambassador from the 2014 growing season, S concentration was significantly and positively correlated with Zn concentration and seed dry matter, while Se concentration was significantly and negatively correlated with number of seeds/pod. For Ambassador from the 2015 growing season, a significant and positive correlation was found between Zn concentration and number of seeds/pod and seed dry matter, between pod length and pod perimeter, and between seed dry matter and pod length. For Premium from the 2014 growing season, a significant and positive correlation was observed between S and Zn concentrations, between Se concentrations and pod perimeter, between number of seeds/pod and pod perimeter and pod length, and between pod perimeter and pod length and seed dry matter. In addition, Zn concentration was significantly and negatively correlated with pod length. For Premium from the 2015 growing season, a significant and positive correlation was found between Se concentration and seed dry matter, and between pod length and pod perimeter, while a significant and negative correlation was found between Zn and Se concentrations (Table 4).

Discussion

In the present study, we investigated the effect of foliar-applied selenate and zinc oxide at the flowering stage on two pea varieties. Parameters of growth; concentrations of Se, Zn, and S; and the

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TABLE 2 Effect of foliar-applied Se and Zn, variety, and year on seed dry matter, number of seeds per pod, pod length, and pod perimeter.

Year	Treatment	Seed dry matter (%)			Number of seeds per pod			Pod length (cm)			Pod perimeter (cm)		
		Ambassador	Premium	*p*-value	Ambassador	Premium	*p*-value	Ambassador	Premium	*p*-value	Ambassador	Premium	*p*-value
2014	Control	28.5 ± 1.19^{B}	24.6 ± 0.38^{C}	**<0.001**	5.20 ± 0.98^{A}	5.08 ± 0.85^{B}	0.863	6.54 ± 0.18	5.57 ± 0.27^{A}	**<0.001**	4.13 ± 0.13	4.07 ± 0.09^{AB}	0.542
	Se1	26.6 ± 0.34^{B}	23.6 ± 0.16^{B}	**<0.001**	4.62 ± 0.23^{A}	4.95 ± 0.18^{B}	0.062	6.82 ± 0.20	5.57 ± 0.14^{A}	**<0.001**	4.21 ± 0.12	4.26 ± 0.10^{B}	0.509
	Se2	26.0 ± 1.40^{B}	24.2 ± 0.54^{B}	0.053	4.35 ± 0.25^{A}	5.00 ± 0.05^{B}	**0.002**	6.72 ± 0.23	5.55 ± 0.13^{A}	**<0.001**	4.09 ± 0.05	4.31 ± 0.01^{B}	**<0.001**
	Zn1	21.1 ± 4.98^{A}	23.6 ± 0.17^{BC}	0.343	6.55 ± 0.62^{B}	3.50 ± 0.58^{A}	**<0.001**	6.36 ± 0.64	5.15 ± 0.24^{A}	**0.012**	4.19 ± 0.35	3.88 ± 0.14^{A}	0.143
	Zn2	27.9 ± 0.79^{B}	19.3 ± 0.28^{A}	**<0.001**	6.50 ± 0.49^{B}	3.88 ± 0.57^{A}	**<0.001**	6.53 ± 0.50	5.23 ± 0.33^{A}	**0.005**	4.19 ± 0.10	3.81 ± 0.33^{A}	0.073
	p-value	**<0.005**	**<0.001**		**<0.001**	**0.001**		0.524	**0.046**		0.869	**0.003**	
2015	Control	22.3 ± 0.98^{B}	20.9 ± 0.55^{AB}	**0.049**	4.40 ± 0.89	4.27 ± 0.54	0.812	6.14 ± 0.45	5.70 ± 0.46	0.223	3.44 ± 0.23	3.44 ± 0.02	0.980
	Se1	22.5 ± 0.29^{B}	21.1 ± 0.86^{AB}	**0.018**	4.08 ± 0.39	4.29 ± 0.26	0.414	6.17 ± 0.33	5.63 ± 0.37	0.073	3.59 ± 0.18	3.32 ± 0.05	**0.024**
	Se2	21.9 ± 0.79^{AB}	22.1 ± 0.48^{B}	0.688	4.29 ± 0.54	4.03 ± 0.44	0.471	6.24 ± 0.35	5.66 ± 0.32	**0.048**	3.76 ± 0.10	3.45 ± 0.09	**0.004**
	Zn1	20.6 ± 1.02^{A}	21.3 ± 1.14^{AB}	0.385	3.92 ± 0.83	3.83 ± 0.18	0.824	6.03 ± 0.33	5.29 ± 0.30	**0.015**	3.63 ± 0.13	3.30 ± 0.15	**0.016**
	Zn2	22.6 ± 0.54^{B}	20.2 ± 0.03^{A}	**<0.001**	4.28 ± 0.26	4.63 ± 0.76	0.422	6.35 ± 0.32	5.68 ± 0.49	0.065	3.64 ± 0.11	3.39 ± 0.08	**0.010**
	p-value	**0.013**	**0.034**		0.833	0.231		0.777	0.564		0.131	0.102	
	**p*-value across	**<0.001**	**<0.001**		**0.003**	**0.002**		0.488	0.084		0.446	**<0.001**	

Control: without Se/Zn, Se1: 50 g of Se/ha, Se2: 100 g of Se/ha, Zn1: 375 g of Zn/ha, Zn2: 750 g of Zn/ha; mean ± SD; n = 4. Means within a column followed by different letters are significantly different. p-values in the same row mean the effect of Se/Zn dose. p-values in the same column mean the effect of variety. *p-values across refer to the effect of year. p-values in bold are statistically significant.

species of Se were assessed in seeds. The results highlight that selenate increased seed Se concentration in both pea varieties (Table 1 and Figure 1A) and that the predominant Se species identified in Se-enriched seeds was SeMet (Table 3 and Figure S1). In contrast, zinc oxide had no beneficial effect on seed Zn concentration (Table 1 and Figure 1B). Generally, growth parameters and seed S concentration were not negatively affected by selenate and zinc oxide applications (Tables 1, 2, Figure 1C).

Pea was chosen, as it constitutes an important staple legume, with global significance for food security. Previous studies on foliar Se and Zn fertilization indicated that field peas, mainly due to their higher protein concentration, may be more efficient in Se and Zn uptake and seed accumulation than cereals (Poblaciones and Rengel, 2017; Poblaciones and Rengel, 2018). The two pea varieties were selected based on their high-yielding capacity. Selenium and Zn solutions were administered *via* foliar application, as this approach reduced the impact of soil properties on interactions between the examined minerals (Malka et al., 2022). However, differences between soils in 2014 and 2015, as well as climate differences could have contributed to additional variability in Se or Zn foliar uptake. Selenium as selenate was employed due to its accredited high efficiency for foliar uptake (Ros et al., 2016). Though Se is not considered an essential element for higher plants, the beneficial effects of low doses of Se on plant growth, development, and yield, and enhanced resistance to abiotic stresses have been reported, as reviewed previously (Hasanuzzaman et al., 2020). Zinc oxide was tested due to its recommendation by the agro-industry. To the best of our knowledge, there is a research gap regarding effects of foliar-applied zinc oxide on pea uptake. In contrast to Se, Zn is an essential trace element for the plant, influencing crop yield and quality (Hacisalihoglu, 2020).

Neither selenate nor zinc oxide had pronounced effects on growth parameters in our study (except in part for Zn for number of seeds/pod, Table 2). Previously tested individual and combined foliar application of sodium selenate and zinc sulfate at early seed filling likewise did not produce differences in the growth of pea (Poblaciones and Rengel, 2017). Similarly, another study did not show any increase in growth parameters of pea upon foliar application of selenate and selenite at the flowering stage (Poblaciones et al., 2013). In contrast, Pandey et al. (2013) found that foliar-applied zinc sulfate at bud initiation had a positive effect on the yield parameters of field pea, including number of flowers, number of pods, their size, and seed numbers. It is possible that differences in Zn status at onset contributed to these observations.

The Premium variety generally accumulated greater amounts of Se in seeds than the Ambassador variety upon biofortification in both years (Figure 1A). However, the effect of variety was significant only in the 2015 growing season, despite the fact that seed Se concentration in both varieties was not significantly affected by the growing year. The beneficial

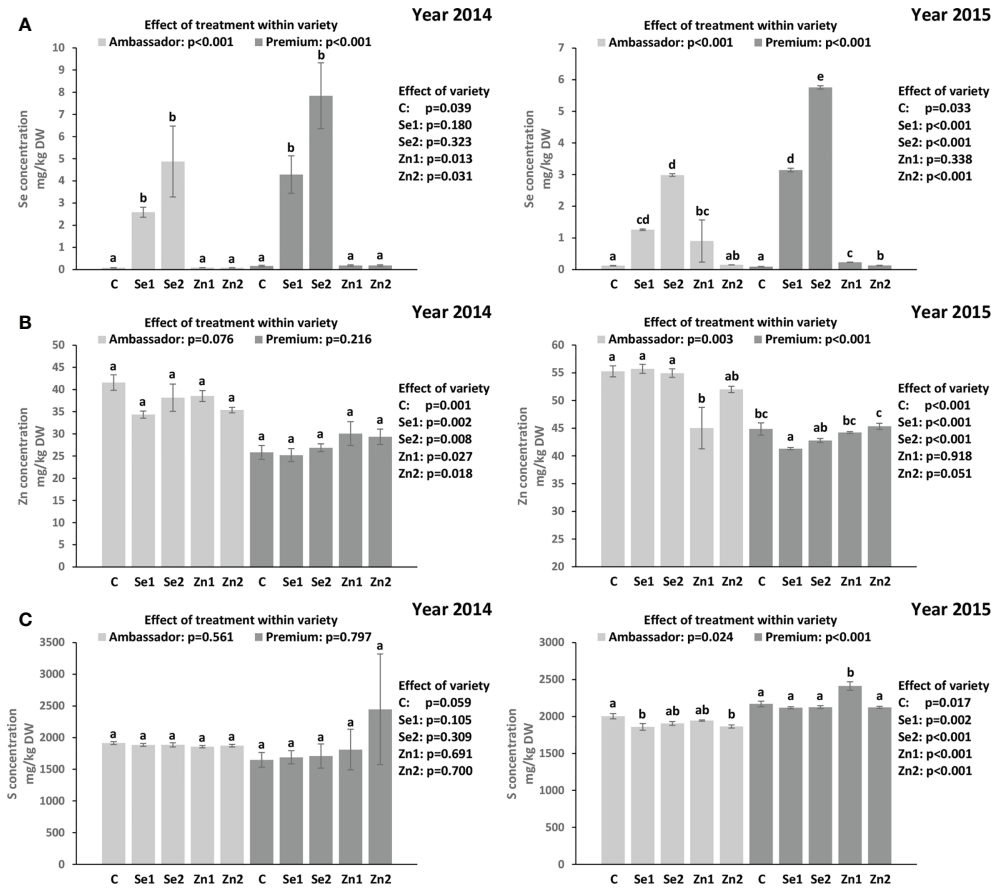

FIGURE 1
Effect of foliar Se and Zn treatments and variety in two growing seasons (2014 and 2015) on the Se concentration **(A)**, Zn concentration **(B)**, and S concentration **(C)** in pea seeds. Control: without Se/Zn; Se1: 50 g of Se/ha; Se2: 100 g of Se/ha; Zn1: 375 g of Zn/ha; Zn2: 750 g of Zn/ha; mean ± SD; n = 4. Bars not sharing the same superscript are significantly different within variety. p-values on the right side of the figure show the effect of treatment across the two varieties (i.e., Ambassador vs. Premium).

effect of foliar-applied selenate on the Se concentration in pea seed is in accordance with previous studies (Smrkolj et al., 2006; Poblaciones et al., 2013; Poblaciones and Rengel, 2017; Poblaciones and Rengel, 2018) and a higher efficiency of foliar-applied selenate than selenite in boosting Se concentration in pea seed was also reported (Poblaciones et al., 2013). The linear and positive trend between foliar selenate treatment and seed Se concentration (Table S1) is further in line with previous studies on pea (Poblaciones et al., 2013; Poblaciones and Rengel, 2018).

The health-related effects of Se are expected to mainly depend on the total amount of Se and the chemical speciation of Se in the food crops. Selenium speciation analysis showed that selenomethionine (SeMet) was the predominant Se species identified in seeds of pea treated with 100 g of Se/ha as selenate, with 40%–76% of total Se (Table 3 and Figure S1). A similar proportion of SeMet (49%–67%) was found in seeds of pea upon foliar application of selenate in a previous study (Smrkolj et al., 2006). In the seeds of chickpea treated with foliar Se, a greater proportion of SeMet was obtained in plots fertilized with selenate (84%–91%), followed by those fertilized with selenite (63%–74%) (Poblaciones et al., 2014). A positive effect of foliar (and soil) application of selenate or selenite on the concentrations of organic Se forms (selenocysteine and selenomethionine) was also observed in lentil seeds (Thavarajah et al., 2015). So far, studies on Se fertilization of legumes indicated that Se concentration and speciation in their seeds may be affected by the method of Se application, Se dose, Se form, plant species and variety, and processing (freezing and cooking) (Smrkolj et al., 2006; Poblaciones et al., 2013; Poblaciones et al., 2014; Poblaciones and Rengel, 2017; Poblaciones and Rengel, 2018).

Selenomethionine is especially beneficial for human and animal health, as it is more bioavailable and less toxic than inorganic Se (Schrauzer, 2003; Rayman, 2004; Zhang et al., 2013). Since higher animals and humans are unable to synthesize SeMet in their organs, and the body incorporates it

TABLE 3 Selenium species concentrations and percentage (given in brackets) of Se species of total μg/g Se in seeds of pea grown with the foliar treatment of 100 g of Se/ha in the form of selenate.

Sample	SeCys (μg/g)	SeMetSeCys (μg/g)	Na_2SeO_3 (μg/g)	SeMet (μg/g)	Na_2SeO_4 (μg/g)	Total Se (μg/g)	Se species recovery (%)
A1	0.15 (2.35%)	0.20 (3.04%)	0.04 (0.69%)	3.73 (57.8%)	0.91 (14.0%)	6.46	78%
A2	0.26 (3.86%)	0.40 (5.89%)	0.12 (1.82%)	5.16 (76.1%)	1.27 (18.8%)	6.78	106%
A3	0.29 (4.70%)	0.77 (12.5%)	ND	4.11 (66.6%)	1.29 (20.9%)	6.16	105%
B1	0.17 (2.96%)	0.06 (0.97%)	0.09 (1.52%)	2.30 (40.1%)	0.93 (16.1%)	5.75	62%
B2	0.18 (3.20%)	0.10 (1.75%)	ND	3.35 (59.3%)	0.92 (16.3%)	5.65	81%
B3	0.22 (3.73%)	0.27 (4.65%)	0.10 (1.62%)	4.17 (70.6%)	1.01 (17.1%)	5.90	98%
B4	0.19 (5.48%)	0.19 (5.68%)	0.06 (1.74%)	2.37 (70.0%)	0.52 (15.3%)	3.38	98%

A1–3, Ambassador variety; B1–4, Premium variety; ND, non-detectable. Recovery expressed as the sum of the Se species (detected by HPLC-ICP-MS vs. total Se determined by ICP-MS).

into the protein pool (Schrauzer, 2003), SeMet is a highly suitable form of Se for nutritional supplementation, food fortification, and biofortification. Such strategies may overcome low Se intakes observed in many countries, including European ones (Stoffaneller and Morse, 2015). Selenomethionine and Se-methylselenocysteine exhibit a strong antioxidant activity and have been widely employed as dietary supplements in the chemoprevention of chronic diseases including cancer, diabetes, and cardiovascular diseases (Zhang et al., 2013; Gómez-Jacinto et al., 2020). Kirby et al. (2008) observed that increases in Se plasma concentrations were much higher (~40%) in a trial group consuming biofortified wheat biscuits that contained a higher SeMet fraction than a group consuming biscuits with a lower proportion of SeMet. In the present study, the high percentage of SeMet in Se-enriched pea seeds suggests that Se in these seeds could be effectively accumulated and transformed into health-promoting Se species.

Unlike Se, our results showed that zinc oxide did not positively affect Zn concentration in pea seeds (Figure 1B). However, the beneficial effects of foliar-applied zinc sulfate on Zn concentration in pea seed were reported previously (Pandey et al., 2013). This may indicate that zinc sulfate is a more suitable form of Zn to be employed in further foliar Zn fertilization studies of pea. However, other Zn forms should also be considered. A recent trial on corn showed that Zn, when foliar-applied in complexed form, both as ZnEDTA and especially as glycine-chelated Zn complex (ZnGly), may pose interesting novel candidates to improve Zn accumulation in the plant, with possible differing release kinetics. They also were of lower phytotoxicity than zinc sulfate (Xu et al., 2022), allowing applications at a wider dose range. ZnGly would also be a source of nitrogen.

When investigating effects on S, selenate had no beneficial impact on its concentration in pea seeds (Figure 1C). Owing to the chemical similarity between Se and S, the availability of S plays a crucial role in Se accumulation due to competitive effects in their absorption, translocation, and assimilation (Abdalla et al., 2020). It is still not clear whether sulfate transporters in non-hyperaccumulators take up S preferentially over Se. Therefore, it has been proposed that Se and S acquisition can influence one another mutually, which was demonstrated by a significant correlation between Se and S tissue accumulations (Abdalla et al., 2020). In contrast, no significant correlations were found between Se and S concentrations in pea seeds. However, significant and positive correlations were observed between Zn and S concentrations in seeds of two pea varieties for one growing season (Table 4), which deserves further investigation, though positive physiological interactions were reported earlier for grains (Cakmak et al., 2010).

In the present study, the significant increase in seed Se accumulation (Figure 1A) indicated efficient absorption and mobility of foliar-applied Se. In contrast, non-significant changes in seed Zn accumulation (Figure 1B) may suggest low absorption or mobility of foliar-applied Zn. It is worth noting that there is still a knowledge gap on the regulation of Se/Zn transport in the plant following foliar Se/Zn application (Cardini et al., 2021). Deciphering these mechanisms is relevant to improve the efficiency of foliar Se/Zn fertilization. This would also be relevant in sight of a potential co-application of Se and Zn, as it is unclear whether uptake occurs *via* the same mechanisms, e.g., involving similar carriers. Such interactions are important in sight of Se and Zn levels in seeds, and eventually for nutritional aspects. Considering the findings regarding Se intake recommendations, consumption of 100 g of seeds of pea

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TABLE 4 Pearson correlation coefficients between seed mineral concentrations and growth parameters evaluated for two pea varieties (Ambassador and Premium) grown in two seasons (2014 and 2015).

Ambassador variety

	Se	Zn	S	NS	PP	PL	DM
Se		0.397	−0.177	0.208	0.438	−0.048	−0.096
Zn	−0.361		−0.046	0.500*	0.039	0.057	0.569**
S	−0.219	**0.668****		0.227	−0.302	−0.198	0.007
NS	−0.566**	0.087	−0.160		−0.258	−0.113	0.302
PP	−0.111	0.064	0.232	0.350		**0.497***	0.179
PL	0.366	−0.158	−0.107	−0.161	0.158		0.524*
DM	0.012	0.123	0.494*	−0.270	0.294	0.083	

Premium variety

	Se	Zn	S	NS	PP	PL	DM
Se		−0.609**	−0.382	−0.142	0.185	0.106	0.536*
Zn	−0.356		0.301	0.220	0.163	0.302	−0.282
S	−0.229	**0.706****		−0.283	−0.135	−0.169	0.038
NS	0.418	−0.317	0.075		0.182	0.427	−0.354
PP	0.617**	−0.308	0.004	0.702**		**0.562****	−0.122
PL	0.356	−0.485*	0.026	0.757**	0.587**		−0.190
DM	0.326	−0.275	−0.387	0.412	0.466*	0.348	

NS, number of seeds per pod; PP, pod perimeter; PL, pod length; DM, seed dry matter. Level of significance, *$p < 0.05$, **$p < 0.01$.
In light gray—2014 growing season, in dark gray—2015 growing season. In bold—effects consistently significant over two varieties.

TABLE 5 Selenium intake and percentage of recommended dietary allowance for Se (% RDA) covered by 100 g of pea seeds.

Variety/ Year	Se treatment (g of Se/ha)	Se intake form 100 g (µg/day)	% RDA from 100 g (USDA*)	% RDA from 100 g (EFSA*)
Ambassador				
2014				
	0	8	15	11
	50	259	471	370
	100	487	885	696
2015				
	0	12	22	17
	50	126	229	180
	100	299	544	427
Premium				
2014				
	0	16	29	23
	50	428	778	611
	100	784	1,425	1,120
2015				
	0	9	16	13
	50	314	571	449
	100	576	1,047	823

*55 and 70 µg RDA (recommended dietary allowance) and AI (adequate intake) according to USDA and EFSA, respectively.

biofortified by 50 and 100 g of Se/ha would be a good source of Se, although it would already exceed the RDA for Se (55 µg, Table 5).

Conclusions

In summary, the present study highlighted that selenate and zinc oxide had no marked negative effects on growth parameters of pea varieties, in line with a lack of toxic effects. The lack of beneficial effects of zinc oxide on seed Zn accumulation suggests that future studies on pea should focus on more readily available forms of Zn, such as zinc sulfate. In contrast, selenate substantially improved seed Se accumulation in both varieties with increasing Se dose. However, selenate had no beneficial influence on seed S accumulation, which may suggest no perturbed amino acid regulation in pea.

Small amounts of pea biofortified with 50 g of Se/ha would cover the RDA of Se; however, a very high intake may not be recommended, as the UL could be reached (400 µg). Lower selenate doses could be employed in future studies. Also, evaluating the impact of climate conditions on the investigated parameters warrants further experiments under field conditions, which was not the aim of the present study. Our study highlights the effectiveness of foliar biofortification of pea with Se, which could be a promising strategy to improve human nutrition.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

MM designed and carried out the experiments, as well as prepared the first version of the manuscript. TB was involved in the supervision of several analyses, as well as statistical interpretation and manuscript writing. JL carried out analyses of selenium speciation. GL was involved in the conceptualization, supervision of the project and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Beyond antioxidants: Selenium and skeletal muscle mitochondria

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The element, Selenium (Se), has an essential nutritive and biological role as a trace mineral known primarily for its vital antioxidant functions as a constituent of the selenoenzyme, glutathione peroxidase. However, Se also has a much more global biological impact beyond antioxidant function. The objective of this review is to present an overview of prior research on the extra-antioxidant effects of Se with a key focus on skeletal muscle mitochondrial energetics. Cognizance of these additional functions of Se is requisite when formulating and recommending dietary supplementation of Se in humans or animals. Chief amongst its myriad of biological contributions, Se influences mitochondrial capacity and function and, subsequently, muscular health. Dietary Se supplementation has been shown to increase skeletal muscle mitochondrial volume density and within some cell lines, Se treatment increases mitochondrial biogenesis and respiratory capacity. In addition, the selenoproteins H, N, W, and O and deiodinases exhibit varying effects on mitochondrial and/or skeletal muscle function. Selenoprotein H enhances mitochondrial biogenesis whereas selenoproteins N and W appear to influence muscle calcium homeostasis which impacts mitochondrial function. Moreover, selenoprotein O's intramitochondrial residence facilitates Se's redox function. Deiodinases regulate thyroid hormone activation which impacts muscle cell regeneration, metabolism, and reactive oxygen species production. Although the precise relationships between dietary Se and skeletal muscle mitochondria remain unclear, previous research constitutes a firm foundation that portends promising new discoveries by future investigations.

KEYWORDS

selenium, mitochondria, mitochondrial biogenesis, selenoprotein, skeletal muscle

Introduction

Elemental selenium (Se) was discovered in 1817 by Jöns Jacob Berzelius who happened upon the element when analyzing an unknown impurity present in manufactured sulfuric acid samples (1). He named the compound selenium after the Greek word for moon, *selene*. Upon its discovery, Se was added to the periodic table as atomic number 34 with a molecular weight of 78.971 Da. Since the observance of Se in 1817, numerous reports have revealed its necessity for maintenance of several bodily

functions. As such, Se is now considered an essential mineral for humans and animals. Importantly, acute or chronic Se toxicity and deficiency can occur at high and low dietary intake levels, respectively, both of which are accompanied by negative health outcomes. Conversely, balanced dietary Se intake is highly beneficial and well known for its vital antioxidant properties.

Dietary Se can be found in inorganic (e.g., selenite and selenate salts) and organic (e.g., selenomethionine, selenocysteine, and Se-enriched yeast) forms, and the absorption and metabolism of Se differs between the forms. Bioavailability of inorganic Se may be lower than organic Se. Dairy cows fed 3 mg Se/d in the form of Se-yeast had a greater increase in Se concentration in whole blood and had numerically elevated Se concentrations in skeletal muscle, heart, and liver compared to those fed 3 mg Se/d in the form of sodium selenite (2). Rats fed 1, 2, or 4 mg Se/kg diet as selenomethionine had greater Se concentrations in muscle compared to rats fed the same amount of Se as sodium selenite (3). In first parity gilts, Se-yeast supplementation resulted in greater serum Se concentrations at breeding and 90 d post breeding than sodium selenite supplementation, but not at weaning (4). However, Se-yeast supplementation did result in a greater Se content in the loin of the gilts and their progeny immediately after birth and at weanling age (4). Thus, the form of dietary Se may impact the availability of Se and the incorporation of Se into tissues which is important to consider when evaluating results of published works. Regardless of species, Se toxicity must also be considered when determining the form of dietary Se. Organic Se supplementation has been shown to be less toxic than inorganic forms such as groundwater selenate, which has a higher toxicity and should be avoided (5). However, organic Se toxicity is still a potential concern. Some studies suggest intakes >90 μg Se/day (in humans) were associated with greater incidences of diabetes, skin cancer, and prostate cancer (5). More research is encouraged to determine quantitative recommendations of each source of dietary Se to optimize health and performance outcomes.

One of Se's most well-known biological roles is as a constituent of several glutathione peroxidase (GPx) isozymes which have an array of functions, including protecting against oxidative stress, minimizing inflammation, and regulating cell death. Examples include GPx1, the most abundant and ubiquitously expressed isozyme which functions to detoxify hydrogen peroxide (H_2O_2) to nontoxic water (H_2O) and GPx4, which has a strong affinity for lipid hydroperoxides and regulates ferroptosis (6). Colloquially known as the "powerhouse of the cell," mitochondria are the primary source of cellular energy, or adenosine triphosphate (ATP), in the body. During aerobic ATP production, electrons are transferred along a network of protein complexes (I–IV) within the inner mitochondrial membrane known as the electron transfer system (ETS; Figure 1). The movement of electrons along the ETS allows protons to be pumped into the intermembrane space, creating an electrochemical gradient which powers complex V, or ATP synthase. The movement of electrons is not without fault; as a normal byproduct of aerobic ATP production, electrons may leak from the ETS before reaching the final electron acceptor, oxygen. These free electrons may bind with an unpaired oxygen molecule, creating the primary reactive oxygen species (ROS), superoxide (O_2^-). Within the mitochondria, leaking electrons resulting in ROS production occurs predominantly at complexes I and III of the ETS in a resting state, though superoxide may also be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (7). When ROS production overwhelms antioxidant capacity, oxidative stress occurs. The GPx family of enzymes plays a critical role in the prevention of ROS-induced oxidative stress, especially in situations of high stress or energy demand such as during an inflammatory response to injury or pathogens. Thus, both Se and the mitochondria are key biological modulators of skeletal muscle energetics and oxidative stress.

While the antioxidant capabilities of Se have been a popular and integral subject of investigation, there are a vast array of additional biological roles of Se, some which may be extensions of antioxidant activity but others which are unrelated to Se's redox role that may be overlooked. In fact, dietary Se may serve to promote mitochondrial biogenesis, and Se is a component of at least 25 different selenoproteins which have a wide variety of physiological activities. Identification of these other branching properties of Se serves to provide a more comprehensive view of the benefits of dietary Se. Although kidneys have the highest concentration of Se (relative to wet weight), skeletal muscle contains approximately 50% of the body's total Se (8). Therefore, it may be especially important to investigate the role(s) of Se within skeletal muscle. The aim of this review is to

Abbreviations: ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; ATP, Adenosine triphosphate; CaM, Calmodulin; CaMKIV, Calmodulin-dependent protein kinase IV; CnA, Calcineurin A; CREB, cAMP response element-binding protein; D2, Type 2 deiodinase; D3, Type 3 deiodinase; DHPR, Dihydropyridine receptor; DM, Dry matter; ER, Endoplasmic reticulum; ERR, Estrogen-related receptors; ETS, Electron transfer system; $FADH_2$, Flavin adenine dinucleotide; GPx, Glutathione peroxidase; MAM, Mitochondria-associated membrane; MAPK, Mitogen-activated protein kinase; MyHC, Myosin heavy chain; NADH, Reduced nicotinamide adenine dinucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; NRF, Nuclear respiratory factor; PGC-1α, Proliferator-activated receptor-γ coactivator-1α; Pi, Inorganic phosphate; PPARα, Peroxisome proliferator-activated receptor α; ROS, Reactive oxygen species; Se, Selenium; SELENOH, Selenoprotein H; SELENON, Selenoprotein N; SELENOO, Selenoprotein O; SELENOW, Selenoprotein W; SERCA, Sarco/endoplasmic reticulum Ca^{2+} transport ATPase; SOD2, Superoxide dismutase 2; SR, Sarcoplasmic reticulum; T3, 3,5,3′-triiodothyronine; T4, Thyroxine; Tfam, Mitochondrial transcription factor A; TH, Thyroid hormone; TSH, Thyroid stimulating hormone; UCP3, Uncoupling protein 3.

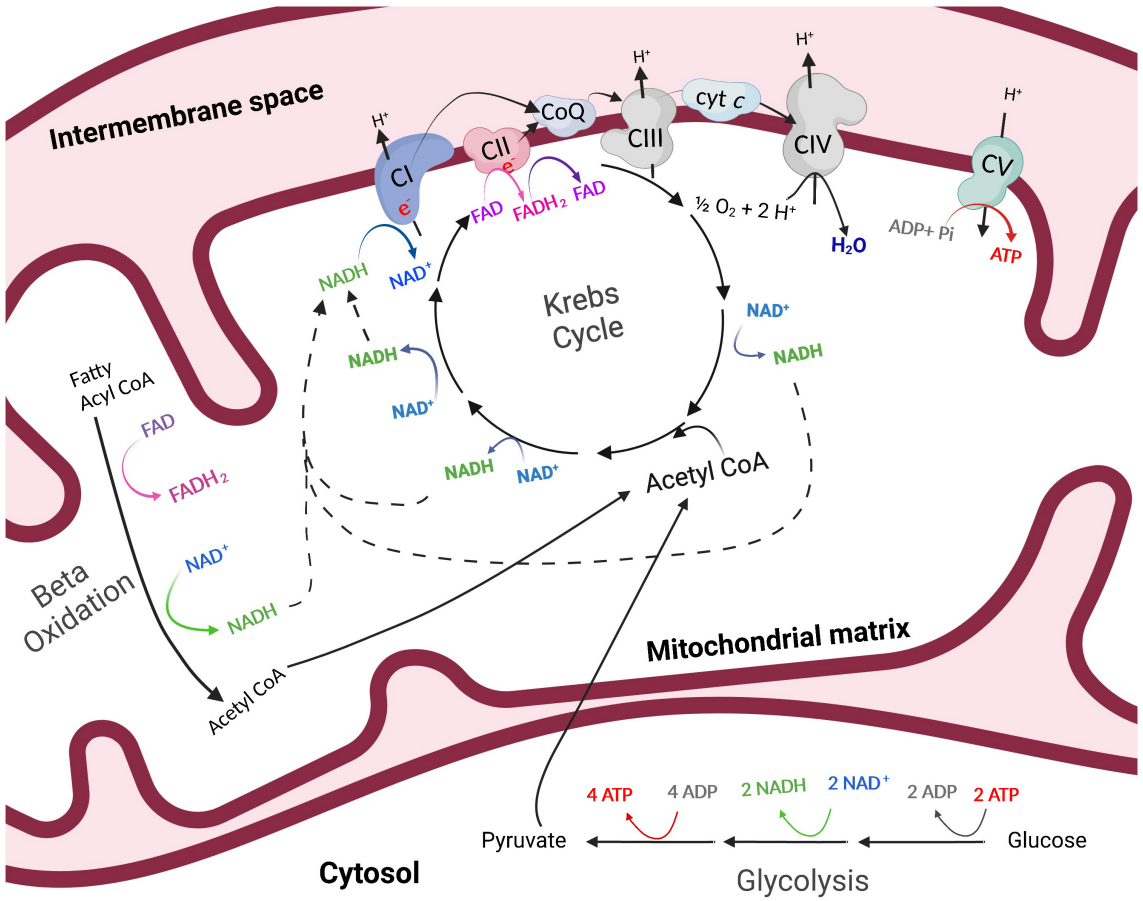

FIGURE 1
The process of energy production outside of (glycolysis) and within (oxidative phosphorylation) the mitochondria. Through anaerobic glycolysis, glucose is metabolized in the cytosol of the cell to pyruvate. Pyruvate may then be transported into the mitochondria where it is oxidized to acetyl CoA, which enters the Krebs cycle in the mitochondrial matrix. The byproducts of the Krebs cycle, NADH and $FADH_2$, provide high-energy electrons to the electron transfer system which resides in the inner mitochondrial membrane. As electrons are transferred, protons are pumped into the intermembrane space of the mitochondria and a proton gradient is generated. Protons then flow through ATP synthase, or complex V (CV) of the electron transfer system, following the chemical gradient to combine with adenosine diphosphate (ADP) and inorganic phosphate (Pi), creating ATP. This process is referred to as oxidative phosphorylation. Created with Biorender.com.

characterize the influence of Se on muscle function as it relates to mitochondrial energetics. We intend to demonstrate several lesser-known ways Se is critically intertwined in skeletal muscle mitochondrial health.

Mitochondrial biogenesis and capacity

Mitochondria are essential organelles for many biological processes due to their role as generators of ATP. The process by which mitochondria produce ATP is known as oxidative phosphorylation (Figure 1). Glucose is metabolized in the cytosol of the cell to pyruvate. In the presence of oxygen, pyruvate is then transported into the mitochondria where it is oxidized to acetyl CoA, which enters the Krebs, or citric acid cycle in the mitochondrial matrix. The byproducts of the Krebs cycle, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), provide high-energy electrons to the ETS which resides in the inner mitochondrial membrane. As electrons are transferred across the complexes of the electron transfer system, protons are pumped into the intermembrane space of the mitochondria and a proton gradient is generated. Protons then flow through ATP synthase along the chemical gradient to combine with adenosine diphosphate (ADP) and inorganic phosphate (Pi), creating ATP (Figure 1). Importantly, mitochondria play pivotal regulatory energetic roles that determine cell growth, metabolism, stress responses, and even cell death.

A scarce amount of previous research has yielded intriguing results showing that Se supplementation increases skeletal muscle mitochondria. Horses that received 0.3 mg Se/kg dry

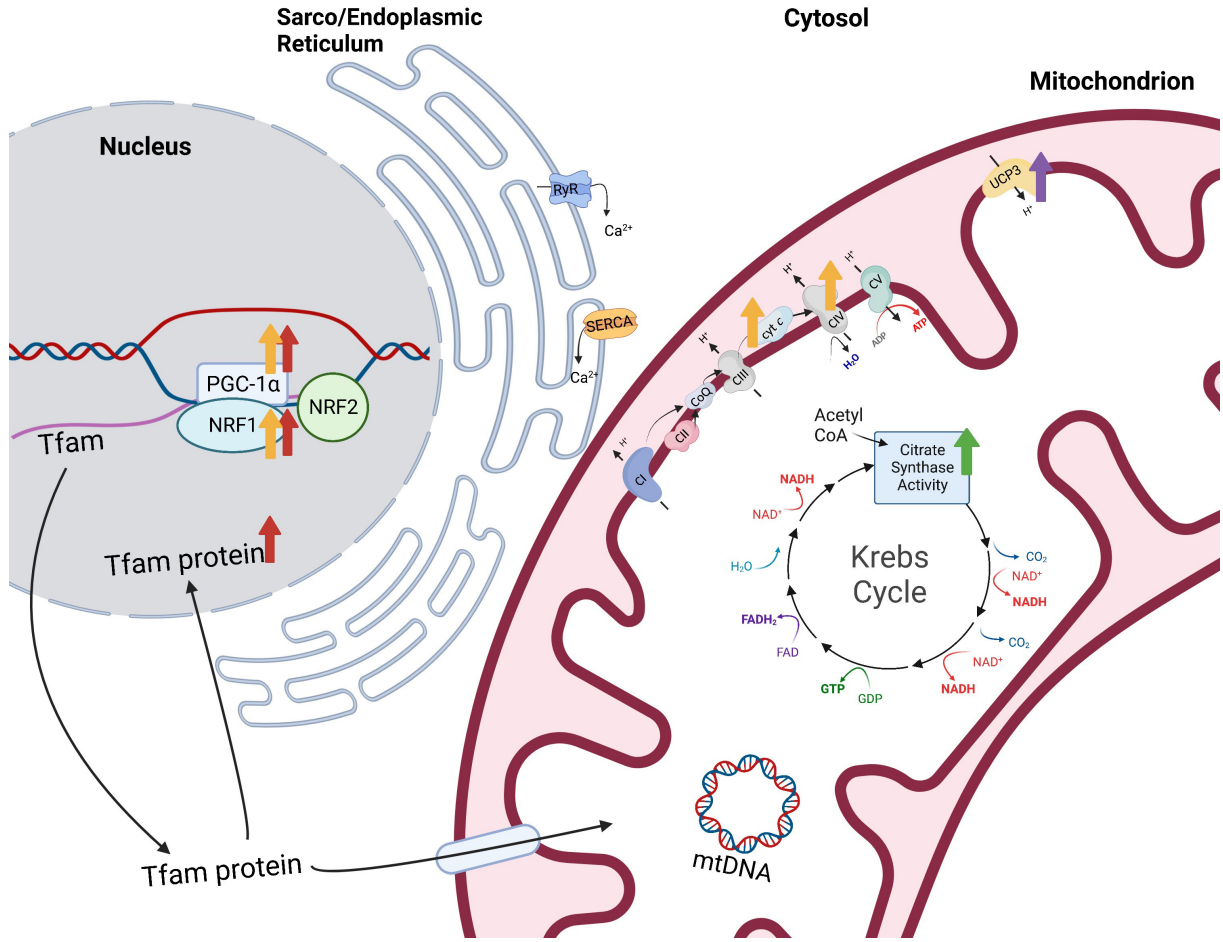

FIGURE 2
Impacts of selenium supplementation or treatment on mitochondrial function. Citrate synthase activity, a measurement of mitochondrial volume density, is greater (green arrow) in the gluteus medius muscle of horses supplemented 0.3 mg Se/kg DM (9). Additionally, overexpression of the *selenoprotein H* (*SELENOH*) gene in murine hippocampal HT22 neuronal cells resulted in greater levels of mitochondrial biogenesis regulators proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) and nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (Tfam) within the nuclear fraction [red arrows (10)]. Similarly, sodium selenite-treated HT22 neuronal cells also had greater PGC-1α and NRF1 and greater mitochondrial proteins, cytochrome *c* and cytochrome *c* oxidase IV [yellow arrows (11)]. Finally, type 2 deiodinase (D2) induction in C2C12 cells resulted in increased expression of mitochondrial *uncoupling protein 3* [*UCP3* (12)]. Created with Biorender.com.

matter (DM) had greater citrate synthase activity in the gluteus medius muscle at rest compared to horses supplemented with 0.1 mg Se/kg DM [Se supplemented as Se-yeast; Figure 2 (9)]. However, cytochrome *c* oxidase activity was unaffected by Se supplementation. Citrate synthase activity is a commonly used proxy for mitochondrial volume density while cytochrome *c* oxidase activity provides a measure of mitochondrial function (13, 14). Therefore, these results indicate that horses had greater mitochondrial volume density after Se supplementation but no apparent change in mitochondrial function. Somewhat conflictingly, young, male human subjects supplemented 180 μg/d of selenomethionine while endurance training for 10 wk had a lesser increase in vastus lateralis muscle mitochondrial content than non-supplemented individuals in training (15). However, Se supplementation while endurance training caused a greater increase in the size of the individual mitochondria whereas training alone resulted in an increase of the number of mitochondria (15). This suggests a preservation of existing mitochondria due to Se supplementation rather than resynthesis of more small, less mature mitochondria. Importantly, with training, ROS serve as signaling molecules to drive muscle adaptation and, as an antioxidant, Se might limit ROS signals for mitochondrial adaptation. Additionally, the impact of exercise training in combination with Se supplementation may yield varying results dependent upon the intensity of the training protocol. However, in the equine study mentioned above,

horses supplemented Se had an increase in skeletal muscle mitochondrial volume density regardless of whether they were untrained or undergoing submaximal training (9). Impacts of dietary Se supplementation on mitochondrial biogenesis *in vivo* during various exercise training regimes warrants further investigation.

It is possible that size and number of mitochondria increase with Se supplementation due to the antioxidant activity of Se protecting cells and organelles from oxidative stress. Supplementation of sodium selenite in mice protected kidneys from cadmium-induced oxidative stress and prevented apoptosis through mitochondrial pathways (16). Specifically, supplementation inhibited mitochondrial membrane potential collapse, cytochrome *c* release, and caspase activation, and prevented a decrease in voltage dependent anion channels, all of which inhibit mitochondrial signaling of cell apoptosis. This signifies that dietary Se might prevent oxidative stress-induced cell death which likely aids in maintaining a larger volume density of mitochondria.

Alternatively, other research presents the possibility that Se induces mitochondrial biogenesis, or the process of increasing mitochondrial cell numbers from pre-existing mitochondria. Overexpression of the *selenoprotein H* (*SELENOH*) gene in murine hippocampal HT22 neuronal cells resulted in greater levels of the "master regulator of mitochondrial biogenesis," peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α), as well as nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (Tfam) within the nuclear fraction compared to vector-transfected cells [Figure 2 (10)]. In addition, mitochondrial biogenesis regulators correlated with greater mitochondrial mass in the *SELENOH* overexpressed cells. Similarly, sodium selenite-treated HT22 neuronal cells also had greater PGC-1α and NRF1 and greater mitochondrial proteins, cytochrome *c* and cytochrome *c* oxidase IV [Figure 2 (11)]. PGC-1α is known as the master regulator of mitochondrial biogenesis because it induces the activation of multiple transcription factors which compensates for the inability of PGC-1α to bind to DNA. These transcription factors include NRF1 and 2, estrogen-related receptors (ERR) α and γ, and peroxisome proliferator-activated receptor α (PPARα). NRF1 and 2 provide transcriptional control of mitochondrial biogenesis associated genes through contact with the promoter of Tfam (17), while ERRα and γ are stimulated to promote ATP uptake and transport across mitochondrial membranes (18), providing energy for expansion of the mitochondrial network. PPARα is co-activated by PGC-1α to regulate fatty acid oxidation and transportation of proteins responsible for linking beta oxidation with mitochondrial biogenesis (19). Additional transcription factors including glucocorticoids, thyroid hormone, and uncoupling proteins play important roles in mitochondrial biogenesis through coactivation by PGC-1α.

During exercise, PGC-1α can be activated through two primary pathways: calcium (Ca^{2+}) calmodulin-dependent protein kinase IV (CaMKIV)/calcineurin A (CnA) and cAMP response element-binding protein [CREB; Figure 3 (20)]. CaMKIV and CnA are considered Ca^{2+} sensitive enzymes that respond to elevated intracellular levels of Ca^{2+} which are present during muscle contraction. In the presence of increased levels of Ca^{2+}, calmodulin (CaM) will bind and rapidly travel to the nucleus to signal CaMKIV (21). Once activated, CaMKIV will phosphorylate CREB at the Ser133 site (22). Importantly, CaMKIV phosphorylation at this site has been found to be the immediate pathway to creating phosphorylated CREB (pCREB), which occurs directly after potassium depolarization in the contracting muscle. However, a secondary pathway, the mitogen-activated protein kinase (MAPK) pathway, has been demonstrated to be a prolonged kinase of CREB (23, 24). One study examined the time of recruitment for each kinase after a depolarizing stimulus was applied to neurons and found that (1) CaMKIV was predominantly active at 0 to 10 min; (2) both kinases were active at 30 min; and (3) MAPK was the main kinase at 60 min (23). It was concluded that this was the result of the immediate response of calmodulin to lower concentrations of Ca^{2+} when compared to MAPK regulators. Upon formation of the nuclear transcription factor, pCREB, the CREB binding protein (CBP) is recruited and phosphorylated at Ser301 (25). Then, CBP phosphorylation facilitates formation and stabilization of the preinitiation complex through its interaction with a variety of transcription factors (26). Meanwhile, pCREB interacts with a CREB binding site on the promoter of the *PGC-1α* gene to aid in transcription (27). PGC-1α acts as a coactivator as it interacts with NRF1 and NRF2 in addition to ERRs and Tfam, all of which are responsible for transcribing genes that increase electron transport subunits, mitochondrial DNA, and mitochondrial proteins, thus being the primary regulator of mitochondrial biogenesis (28–31).

In addition to increasing mitochondrial biogenesis, Se appears to increase mitochondrial respiratory capacity. When incubated with sodium selenite or selenomethionine, placental tissue and Swan-71, JEG-3, and BeWo trophoblast-like cells exhibited increased respiratory capacity (32). The Se treated cell lines also exhibited greater SELENOH content. Correspondingly, both overexpression of *SELENOH* and sodium selenite treatment in murine hippocampal HT22 neuronal cells resulted in greater oxygen consumption compared to control cells (10, 11). Furthermore, *SELENOH* overexpression prevented a decrease in mitochondrial respiration following UVB-irradiation (10). While these studies were conducted *in vitro*, a recent study in horses investigated the potential for dietary Se to enhance skeletal muscle mitochondrial respiration *in vivo* (33). The results indicated that removing commonly added levels of vitamin E to performance horse diets reduced mitochondrial respiratory capacities but the impairment was rescued by providing horses with 0.3 mg Se/kg DM *via* a proprietary Se yeast blend (33).

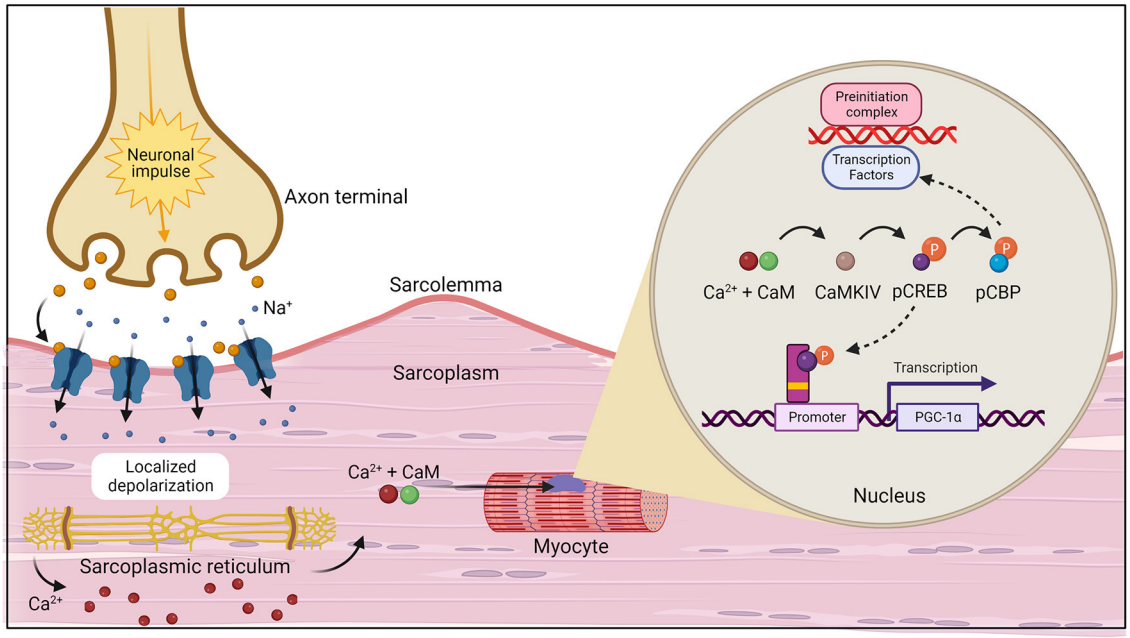

FIGURE 3
The initiation of muscle contraction followed by the two primary pathways, calcium (Ca^{2+}) calmodulin-dependent protein kinase IV (CaMKIV)/calcineurin A (CnA) and cAMP response element-binding protein (CREB) that activate the *PGC-1α* gene which act to increase mitochondrial biogenesis (20). Created with Biorender.com.

Importantly, these studies indicate a functional benefit of Se since mitochondrial respiration drives necessary energy production. Further investigation is required to adequately characterize the relationship between Se and mitochondrial biogenesis and capacity, and to determine the mechanisms by which Se enhances mitochondrial respiration.

Selenoproteins

Many biological effects of Se are mediated by Se-containing proteins, also known as selenoproteins, which generally contain at least one selenocysteine (a Se-containing amino acid and the main biological form of Se) and often serve oxidoreductase functions. This review will discuss four known selenoproteins that may mediate some facet of skeletal muscle mitochondrial function: SELENOH, Selenoprotein N (SELENON), Selenoprotein W (SELENOW), and Selenoprotein O (SELENOO). Additionally, we briefly review the selenoproteins type 2 and type 3 deiodinases (D2 and D3) due to their influence on skeletal muscle regeneration, myogenesis, and metabolism. Previous reports present conflicting results regarding the effect of dietary Se on production of selenoproteins. Broiler chicks supplemented Se *via* sodium selenite had greater expression of *selenoprotein N1, W1,* and *O* in the pectoral muscle compared to non-supplemented chicks (34). However, in rats, only *Sepw1* expression was highly regulated within muscle with sodium selenite and Se-deficiency (35). Therefore, it remains unknown the extent to which se supplementation can impact production and function of the four selenoproteins outlined in the current review. Nevertheless, research does identify multiple integral functions of these selenoproteins within muscle and mitochondria.

Selenoprotein H

Selenoprotein H was initially identified using bioinformatics methods developed around selenocysteine insertion sequence (SECIS) elements of human genomes (36). The SECIS elements allow selenocysteine to be cotranslationally incorporated into the polypeptide; this allows SECIS elements to be utilized, in part, to identify novel selenoproteins. Since then, early study of SELENOH in zebrafish showed localization in the brain ventricular zone, the branchial arches and pectoral fin buds, and the proliferative zone of the retina. Sequence analysis suggests SELENOH has a redox function and resides in the nucleus (37). Subsequently, *via* Western blot and immunohistochemistry, SELENOH was found in the nucleolar fraction specifically within the nucleoli (37). As detailed in the Mitochondrial biogenesis and capacity section of this review, *SELENOH* expression in neuronal cells appears to play a pivotal role in mitochondrial biogenesis and respiratory capacities. Specifically, overexpression of the *SELENOH* gene

in neuronal HT22 cells resulted in greater mitochondrial mass, mitochondrial biogenesis regulators, and oxygen consumption compared to vector-transfected cells (10). Additionally, total and phosphorylated protein kinase A, Akt/protein kinase B, and CREB were significantly increased in SELENOH transfected cells compared to vector transfected neuronal HT22 cells (38). Importantly, CREB senses insufficient energy and may enhance *PGC-1α* transcription to then upregulate mitochondrial biogenesis (20, 39). Protein kinase A and Akt regulate CREB. Alternatively, SELENOH might have antioxidant functions since overexpression of human *SELENOH* in HT22 cells protected against UV-induced cell death by decreasing superoxide levels (40). Selenoprotein H appears to be involved in mitochondrial biogenesis and antioxidant function within neuronal cells. This remains to be investigated within skeletal muscle cells but the potential impact of SELENOH on mitochondrial function is worthy of future consideration.

Selenoprotein N1

Selenoprotein N was originally characterized by bioinformatics methods in 1999 (41) and was implicated in neuromuscular diseases, specifically rigid spine syndrome, not long after the discovery (42). Many of these neuromuscular diseases are now considered selenoprotein N1 (SELENON also referred to as SEPN1)-related myopathies caused by mutations in the *SELENON* gene which occurs mainly in humans. Selenoprotein N1 is a sarcoplasmic reticulum (SR) transmembrane glycoprotein with a cysteine-selenocysteine active site and an N terminus which is exposed to the cytoplasm. Increased fat mass, decreased global body mass, and increased energy expenditure are noted in *SELENON* knockout mice compared to wild-type mice suggesting SELENON is involved in bioenergetics (43). Mutation of the *SELENON* gene in humans may also result in early truncal hypotonia, neck weakness, progressive scoliosis, and a 12% smaller mean diameter of slow-twitch type I muscle fibers compared to fast-twitch type II fibers (44). Ultimately, these results demonstrate that SELENON is integral in muscle function and metabolism. However, the mechanisms that cause these specific muscular impairments with SELENON loss are still relatively unknown.

Selenoprotein N1 serves to regulate Ca^{2+} and redox homeostasis by interaction with and activation of the sarco/endoplasmic reticulum Ca^{2+} transport ATPase 2b [SERCA2b; Figure 4 (46, 48)]. Importantly, SERCA pumps dictate the resting cytosolic Ca^{2+} concentrations because SERCA pumps have a high affinity for removal of Ca^{2+} from the cytosol. Within skeletal muscle, release of Ca^{2+} from the SR and subsequent binding of Ca^{2+} to troponin C is required to induce muscle contraction. However, removal of Ca^{2+} from the cytosol is of equal importance since it functions to stop contraction when necessary. Thus, impairment or loss of SELENON could influence SERCA mediated Ca^{2+} homeostasis within muscles, likely impairing the re-uptake of Ca^{2+} into the SR and limiting muscles from returning to a resting, non-contracted state. Conversely, when stimulated with 100 mM caffeine, flexor digitorum brevis myofibers from SELENON deficient mice had reduced amplitude of Ca^{2+} release compared to wild-type mice which suggests that the ryanodine receptor of the SR may be impaired with loss of SELENON (45). The ryanodine receptor is involved in release of Ca^{2+} from the SR so, although it is evident that SELENON loss results in impairment of Ca^{2+} homeostasis, the origin of this defect remains unclear and may involve both the release (ryanodine receptor) and re-uptake (SERCA) of Ca^{2+}.

The actions of SELENON are also thought to influence mitochondrial function because SELENON is localized near mitochondria-associated membranes (MAMs) of the SR. Along with the SR, mitochondria assist in maintaining Ca^{2+} homeostasis. The MAMs serve several functions, one of which being that the SR and mitochondria can transfer Ca^{2+} at MAMs [Figure 4 (49)]. Within skeletal muscle, SR and mitochondria locally transfer Ca^{2+} in response to caffeine stimulation (50). Physiological concentrations of Ca^{2+} regulate multiple mitochondrial enzymes (51–54) and Ca^{2+} can increase maximum velocity of oxidative phosphorylation by activating many aspects of the oxidative phosphorylation pathway (55). Cells deficient in SELENON had lower ER Ca^{2+} and less Ca^{2+} transport into the mitochondria (43) potentially influencing mitochondrial oxidative phosphorylation.

Absence of SELENON in mice resulted in decreased mitochondrial respiratory capacities in the tibialis anterior, diaphragm, and quadriceps muscles compared to wild-type mice (43). SELENON-devoid cells also had reduced ATP content, slightly decreased mitochondrial membrane potential, reduced complex I activity, and impaired function of complexes II and IV of the ETS [Figure 4 (43)]. In support of this, *SELENON* knockout mice have lower blood glucose and faster muscle and liver glycogen depletion in response to exercise (43) potentially indicating an increased reliance on anaerobic glycolytic metabolism. Anaerobic glycolysis is a less efficient form of energy production and occurs outside of the mitochondria while oxidative metabolism is a more efficient form of energy production and is carried out within the mitochondria (Figure 1). Blunted mitochondrial function due to lack of SELENON could induce a shift toward increased use of anaerobic metabolism to produce necessary cellular energy. Some human patients with SELENON-related myopathy have low body mass index as well as abnormal glucose metabolism (56) which might be a functional result of altered SR regulation and mitochondrial metabolism. Ultimately, SELENON is integral in maintaining Ca^{2+} homeostasis but further study is required to elucidate the full influence of SELENON on mitochondrial function and metabolism.

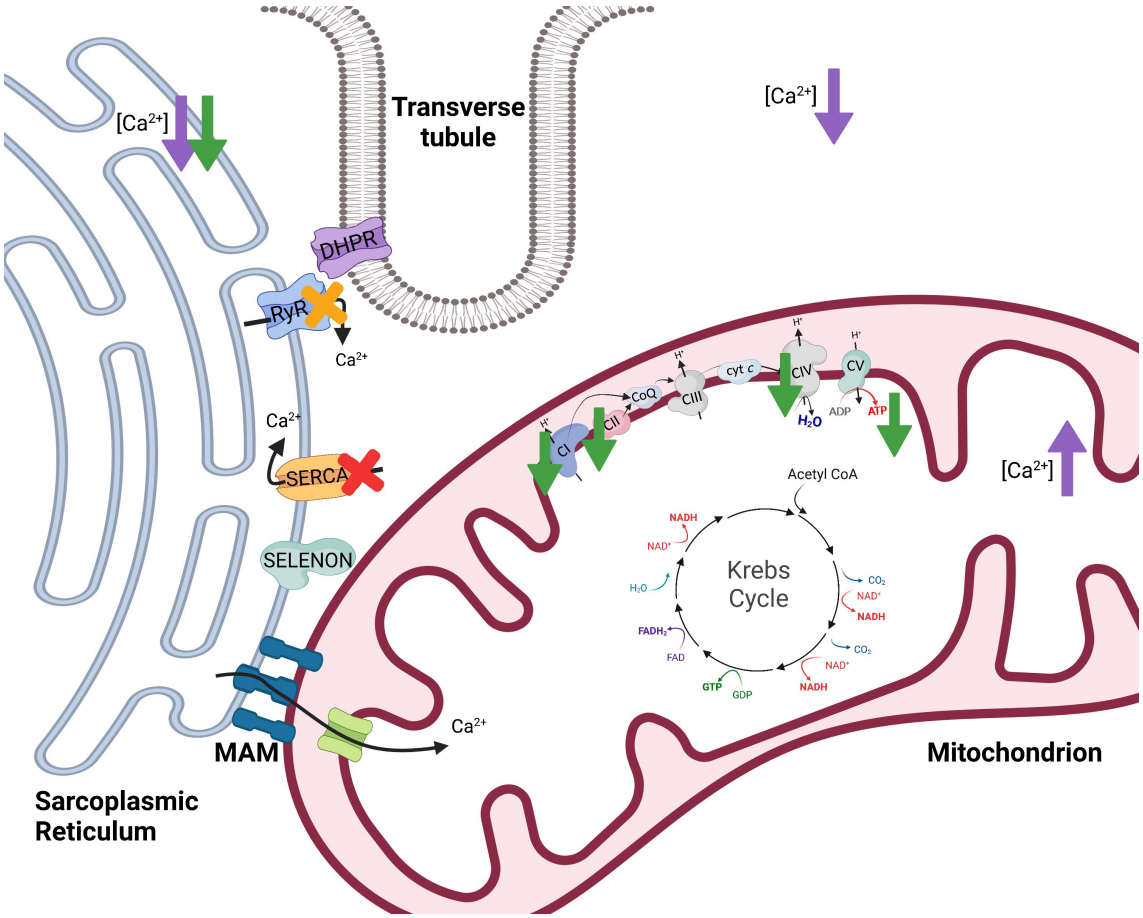

FIGURE 4
The effects of selenoprotein N1 (SELENON) and selenoprotein W (SELENOW) knockout/deficiency in skeletal muscle. To initiate muscle contraction, an action potential travels down the Transverse tubule (T-tubule) to activate voltage-gated channels known as dihydropyridine receptors (DHPR) triggering the ryanodine receptor (RyR) to release Ca^{2+}. When stimulated with 100 mM caffeine, myofibers from SELENON deficient mice had reduced amplitude of Ca^{2+} release which suggests that RyR of the sarcoplasmic reticulum may be impaired with loss of SELENON [yellow X (45)]. Further, SELENON serves to regulate calcium and redox homeostasis by interaction with and activation of the sarco/endoplasmic reticulum calcium transport ATPase (SERCA) 2b [red X (46)]. Cells deficient in SELENON had lower ER Ca^{2+}, less Ca^{2+} transport into the mitochondria, reduced ATP content, reduced complex I (CI) activity, and impaired function of complexes II (CII) and IV (CIV) of the electron transfer system [green arrows (43)]. Lastly, SELENOW deficiency in myoblasts resulted in decreased Ca^{2+} in the cytoplasm and sarcoplasmic reticulum but increased Ca^{2+} levels in mitochondria [purple arrows (47)]. Created with Biorender.com.

Selenoprotein W

One myopathy which is caused by Se deficiency is white muscle disease within young livestock animals. The disease was discovered to be linked to Se deficiency in 1958 (57) which then led to investigation of selenoproteins within muscle and prompted the identification of SELENOW (58, 59). Similar to many of the selenoproteins, SELENOW is highly conserved in mammals. Specifically, SELENOW has been studied in humans, monkeys, rats, mice, sheep, and pigs. Selenoprotein W accumulation is highest within skeletal muscle, heart, and brain but this does not correlate with Se concentrations which are highest within kidneys and lowest in skeletal muscle (60). The exact function(s) of SELENOW remains unknown but SELENOW in rats can bind to glutathione (61) which suggests a redox function. On the other hand, the recombinant form of rat mutant SELENOW in *E. coli* was glutathione bound under anaerobic conditions but not under aerobic conditions indicating SELENOW may exist with or without glutathione (62). There is high expression of SELENOW in proliferating myoblasts but there is minimal expression in differentiated myotubes indicating a potential importance in muscle differentiation (63). Furthermore, SELENOW might regulate Ca^{2+} homeostasis since SELENOW deficiency results in altered Ca^{2+} accumulation and expression of Ca^{2+} channels (47). It is still unclear which functions SELENOW serves, and it is possible that, under different biological conditions, SELENOW has differing functions.

Animals with the peracute form of white muscle disease may present with dysrhythmias, exhaustion, and cardiovascular

collapse while animals with the subacute form may have dysphagia, muscular weakness, muscular pain, and more (64). Since these clinical signs are accompanied by low serum antioxidants, GPx and vitamin E, it has been proposed that white muscle disease is induced by ROS and oxidative stress. However, decreased SELENOW has also been observed in the muscle of animals with white muscle disease. In addition to decreased SELENOW, animals with white muscle disease can have reduced uptake of Ca^{2+} into the SR (65) signifying a potential role of both SELENOW and irregular Ca^{2+} homeostasis in the disease. Total Ca^{2+} levels in the muscle were decreased in broiler chickens fed a Se-deficient diet (47). Within myoblasts, a specific SELENOW deficiency also resulted in decreased Ca^{2+} in the cytoplasm and SR but increased Ca^{2+} levels in mitochondria [Figure 4 (47)]. Mitochondria also exhibited swelling, dilation, disruption of cristae, and decreased mitochondrial membrane potential with SELENOW deficiency. The SELENOW deficient myoblasts had decreased expression of SERCA and ryanodine receptors 1 and 3 (47). Thus, deficiency of SELENOW likely causes impaired ability to release and reuptake Ca^{2+} from the SR which may induce altered Ca^{2+} concentrations and mitochondrial deformities. Ultimately, however, oxidative stress also occurs with Se and SELENOW deficiencies, and the mechanism behind the clinical outcomes of white muscle disease and Se deficiency might be multifactorial. Further investigation could aid in elucidating the exact role of SELENOW in muscle and its influence on mitochondrial function.

Selenoprotein O

Similar to the previously mentioned selenoproteins, SELENOO was originally identified as a selenoprotein using bioinformatics (36). The exact functions of SELENOO, like several other selenoproteins, remain relatively unknown. Presence of a mitochondrial leader sequence in SELENOO, and the localization of SELENOO in the mitochondrial fraction of human embryonic kidney 293T cells indicate that SELENOO likely resides in the mitochondria (66). Additionally, SELENOO might have a redox reaction with another protein through its selenocysteine residue. Selenoprotein O in human embryonic kidney 293T cells is reversibly oxidized when treated with H_2O_2 which also supports the theory of a redox function. It is possible the redox function involves kinase action and regulation of signaling cascades (66). Structural and functional analysis of SELENOO using bioinformatics suggests a three-dimensional fold similar to protein kinases (67). Further, SELENOO transfers adenosine monophosphate (AMP) from ATP to protein substrates (AMPlaytion) that are involved in redox homeostasis (68). Interestingly, SELENOO appears to reside within the mitochondria, but future research is warranted to investigate the significance of its localization and proposed functions.

Deiodinases

Thyroid hormone (TH) serves several critical regulatory functions, including the regulation of satellite cells, or muscle stem cells. Selenoenzymes D2 and D3 are present in skeletal muscle, and their expression influences intracellular TH levels consequently impacting stem cell proliferation and differentiation. The activation, proliferation, fusion, and differentiation of normally quiescent satellite cells is necessary for muscle fiber regeneration following injury. Type 3 deiodinase deactivates thyroxine (T4) and 3,5,3′-triiodothyronine (T3). Specifically, D3 is highly expressed in proliferating myoblasts but lowly expressed in differentiated myoblasts (69). High expression of D3 limits activated TH levels which likely allows for normal satellite cell proliferation and prevention of TH-induced cell apoptosis. Depletion of D3 during *in vivo* muscle regeneration disrupted the regeneration process due to rapid cell apoptosis (69). Conversely, D2 is involved in regulation of muscle fiber regeneration *via* promotion of the differentiation stage. Type 2 deiodinase converts the pro-hormone T4 into T3, the active hormone. *In vitro* and *in vivo*, the absence of D2 limits intracellular T3 concentrations which prevents muscle cell differentiation (70). Thus, both D3 and D2 serve to regulate different stages of muscle satellite cell regeneration [for a full review of D2 and D3 functions, see (71–73)].

The regulation of conversion of T4 to T3 by D2 may impact mitochondria, as well. Low plasma levels of T3, T4, or thyroid stimulating hormone (TSH) stimulate the hypothalamus to release thyrotropin-releasing hormone which, in turn, stimulates the anterior pituitary to release TSH, promoting T4 release from the thyroid. On the contrary, high plasma levels of T3, T4, and TSH serve as negative feedback signals to both the hypothalamus and the anterior pituitary. Knockout of the *Dio2* gene resulted in elevated serum TSH levels due to the lack of negative feedback from elevated T4 (12). This could be critical since cultured equine skeletal muscle fibers treated with 10 mIU TSH showed increased mitochondrial oxidative phosphorylation capacity (74). Further, T3 injections increased PGC-1α protein content in rat skeletal muscle (75). Therefore, increases in D2 may serve to downregulate TSH production and, consequently, mitochondrial biogenesis and/or capacity. This is supported by data showing that inducing D2 enzyme activity and protein expression increased intracellular TH action prompting a net shift from oxidative to glycolytic metabolism. The shift in metabolism was also associated with changes in myosin heavy chain (MyHC) expression toward faster, more glycolytic isoforms, MyHC IIa and IIb. However, the shift to glycolytic metabolism did not alter total ATP production. Interestingly, D2 appeared to support multiple antioxidant functions, including upregulation of expression of the antioxidant, *superoxide dismutase 2* (*SOD2*), as well as mitochondrial *uncoupling protein 3* (*UCP3*; Figure 2) which may facilitate movement of protons back into the mitochondrial

matrix to bind unpaired electrons. In support, D2 induction reduced mitochondrial ROS levels which promoted cell differentiation (76). Through mediating TH activity, D2 and D3 influence both muscle metabolism and satellite cell proliferation and differentiation, and D2 may impact mitochondrial capacities through TSH regulation.

Future directions

Current literature suggests a close link between skeletal muscle mitochondria and Se. It appears that several of the impacts of Se on mitochondrial function may be extensions of the antioxidant properties of Se while other effects of Se stem from the more elusive functions of selenoproteins. Specifically, Se increases mitochondrial biogenesis and function, but this could be, in part, due to antioxidant protection. On the other hand, selenoproteins like SELENON and SELENOW may regulate muscular Ca^{2+} signaling and SELENOO resides in the mitochondria and could serve redox functions. However, the precise mechanisms of the Se-mitochondria relationship remain unknown, therefore it is difficult to surmise specific supplementation recommendations to optimize function of skeletal muscle mitochondria. Additionally, much of the literature reviewed in the present manuscript has investigated the impacts of Se or selenoproteins on mitochondria in cell or rodent models with a few other papers focusing on horses (9, 33), humans (15), or chickens (47). Thus, in humans, livestock, and companion animals there is currently no conclusive understanding of how Se supplementation influences skeletal muscle mitochondrial function beyond antioxidants. To address this, there are several methods and technologies which should be utilized to investigate different aspects of mitochondrial function. Mitochondrial volume density can be measured *via* transmission electron microscopy, cardiolipin and mitochondrial DNA content, citrate synthase activity, and more. Additionally, mitochondrial respiratory capacity can be assessed by respirometry, the measurement of oxygen consumption which occurs during oxidative phosphorylation. There are several systems used to perform respirometry including Clark electrode systems, the Seahorse, and the Oxygraph 2k [for a full review, see (77)]. Future studies may look toward implementation of these measures of mitochondrial volume density and respirometry to assess skeletal muscle mitochondrial function following supplementation of Se under different conditions. Some specific areas of interest include the impacts of Se supplementation on mitochondria during growth and development, exercise training, and stress. Importantly, further investigation of the effect of Se supplementation on skeletal muscle mitochondria could provide prevalent information for future Se treatment to optimize mitochondrial function in various species. Enhancement of mitochondrial energy production could provide numerous benefits such as preventing fatigue within exercising muscle (78).

Conclusion

There is minimal current literature on the effects of Se on skeletal muscle mitochondria beyond its antioxidant role(s). However, several studies provide multiple theories and potential avenues for Se influence on mitochondria. Selenium may increase mitochondrial biogenesis, but the magnitude of this impact could differ by tissue, exercise intensity and/or duration, and source of dietary Se. Selenoproteins have a wide range of functions which are not fully elucidated but could influence mitochondrial function. Some of these potential functions of selenoproteins include promoting satellite cell differentiation, mitochondrial biogenesis, redox functions, and regulating Ca^{2+} homeostasis which influences mitochondrial capacity. This review had the specific focus of outlining the non-antioxidant influence of Se on muscle function as it relates to mitochondrial energetics. Therefore, the authors acknowledge that, due to the scope of this review, there is additional existing literature on the impacts of Se that was regretfully not included in the current paper. Nevertheless, the compelling literature covered in the current review demonstrates several ways in which Se impacts skeletal muscle health and mitochondrial function. These results highlight the importance of future studies to classify the exact mechanisms behind the influence of Se on mitochondria because there is much more to Se beyond antioxidants.

Author contributions

SW-S conceived the subject of the review and provided direction on the content of the manuscript. LW, PS, and SW-S wrote the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

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

Publisher's note

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The use of selenium for controlling plant fungal diseases and insect pests

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The selenium (Se) applications in biomedicine, agriculture, and environmental health have become great research interest in recent decades. As an essential nutrient for humans and animals, beneficial effects of Se on human health have been well documented. Although Se is not an essential element for plants, it does play important roles in improving plants' resistances to a broad of biotic and abiotic stresses. This review is focused on recent findings from studies on effects and mechanisms of Se on plant fungal diseases and insect pests. Se affects the plant resistance to fungal diseases by preventing the invasion of fungal pathogen through positively affecting plant defense to pathogens; and through negative effects on pathogen by destroying the cell membrane and cellular extensions of pathogen inside plant tissues after invasion; and changing the soil microbial community to safeguard plant cells against invading fungi. Plants, grown under Se enriched soils or treated with Se through foliar and soil applications, can metabolize Se into dimethyl selenide or dimethyl diselenide, which acts as an insect repellent compound to deter foraging and landing pests, thus providing plant mediated resistance to insect pests; moreover, Se can also lead to poisoning to some pests if toxic amounts of Se are fed, resulting in steady pest mortality, lower reproduction rate, negative effects on growth and development, thus shortening the life span of many insect pests. In present manuscript, reports are reviewed on Se-mediated plant resistance to fungal pathogens and insect pests. The future perspective of Se is also discussed on preventing the disease and pest control to protect plants from economic injuries and damages.

KEYWORDS

selenium, fungal disease, disease resistance, insect resistance, pest control

Introduction

Mineral nutrients have important benefits for the growth and development of many organisms, and are essential factors influencing plant growth and development (Ahn et al., 2005; Cabot et al., 2013; Crane et al., 2014; Elmer and Datnoff, 2014). The essentiality of Se as a nutrient has been proven for humans and animals only, while for higher plants it is a beneficial element (Hasanuzzaman et al., 2020). Both the organic and inorganic forms of Se are available in nature. The available organic forms are selenocysteine (SeCys), selenocystathionine (SeCysth), and selenomethionine (SeMet), etc., and the inorganic forms are mainly elemental Se, selenide (Se^{2-}), selenite (Se^{4+}; SeO_3^{2-}), and selenate (Se^{6+}; SeO_4^{2-}) (Bodnar et al., 2012). Although Se is not an essential nutrient element for plants, it acts as an antioxidant to improve the tolerance of plants to drought and salt stress (Nawaz et al., 2021; Regni et al., 2021), and it reduces the absorption of toxic metal elements and reduces their oxidation (Jiang et al., 2021), which plays a positive role in plant growth and development and helps to improve the yield and quality of grain (Feng et al., 2015; Andrade et al., 2018). Furthermore, recent studies show that Se can also assist plant resistance to pest and pathogen (Xu et al., 2020; Zang et al., 2022). Since the interactions between Se and viruses/bacteria remain largely underexplored, this review paper focuses on the roles of Se in plants against fungal diseases and insect pests, and on the related mechanisms and novel strategies for the application of Se in crop protection.

Selenium-mediated plant resistance to disease pathogens

Plant resistance to diseases refers to the characteristic or ability of plants to prevent the establishment of diseases ensued by the pathogens (Andersen et al., 2018). There are generally two stages of plant resistance to pathogens: (1) resistance to infection and (2) resistance to parasitism. Table 1 and Figure 1 show the different relationships between Se and fungal diseases reported previously in different studies.

i. Se induces structural and functional changes of soil microbial community to prevent fungal pathogen invasion

Species composition or biodiversity of soil microbial community, functional profiles, and their interactions have been connected to plant soil-borne disease outbreaks (Trivedi et al., 2017; Wang et al., 2017; Xiong et al., 2017). The species diversity of soil microbial community of healthy plants is generally higher than that of infested plants. Se content (≥ 0.4 mg kg^{-1}) in the soil significantly enhanced the microbial diversities and the relative abundance of plant growth promoting rhizobacteria (PGPR). The bioconcentration of Se in plant tissues and the improvement of microbiome diversities are related to the enhancement of plant resistance to pathogen infection, showing that the Se content in the soil could indirectly affect the occurrence and transmission of soil-borne diseases (Liu et al., 2019). Meanwhile, Se can decrease the relative abundance of pathogenic fungi, such as *Olpidium* sp., *Armillaria* sp., *Coniosporium* sp., Microbotryomycetes and Chytridiomycetes (Liu et al., 2019). While Se can inhibit the growth and decrease the relative abundance of fungal pathogens in the soil, it can also improve the biodiversity of beneficial microorganisms in soil (Liu et al., 2019), which might vary from fungal pathogens types, Se concentrations as well as the dominant chemical form(s) of Se.

ii. Se enhances the ability of plants to prevent the invasion of fungal pathogens

As the first line of plant defense, the surface structure can hamper the entry of plant pathogens. However, some pathogens can break through the surface barriers and successfully reach the interior of plant tissues. Most of fungal pathogens can form various specialized structures such as haustoria to penetrate the cell for absorbing nutrients, but the obligate fungi will not penetrate through the plasma membranes of plant cells (Pearson et al., 2009). Such fungi make use of the haustoria or intracellular structures at some locations to release effector proteins, which can be recognized by pattern recognition receptors (PRRs) on the plant cell surface or intracellular resistance (R) proteins of the nucleotide-binding domain and leucine-rich repeat (NLR) class, resulting in deeper and stronger immune effects (Jones and Dangl, 2006; Lyu et al., 2019).

The increase of mesophyll cell density is critically important in enhancing plant photosynthetic capacity (Ren et al., 2019). The low Se concentrated treatment (17 mg L^{-1}) significantly increased the number of mesophyll cells (Feng et al., 2015), which was reportedly helpful in maintaining normal chloroplast structure (Xu et al., 2020). In particular, Se can protect the photosynthetic process from pathogen stress by increasing the chloroplast size and reconstructing chloroplast ultrastructure of rape leaves (Filek et al., 2010). In addition, with Se levels (e.g. 0.1 mg kg^{-1} and 0.5 mg kg^{-1}) in soil, the degree of mitochondrial permeability transition pore was significantly decreased after inoculation with *S. sclerotiorum*, indicating that Se could be helpful in maintaining plant cell structures (Xu et al., 2020). The soil Se treatments (0.1 mg kg^{-1} and 0.5 mg kg^{-1}) also significantly reduce the lesion diameter and the incidence of sclerotinia stem rot caused by *S. sclerotiorum* due to improving the defense ability and antioxidant capacity of rape leaves (Xu et al., 2020). Overall, Se enhances the ability of plants to prevent the invasion of pathogens *via* maintaining the plant cell or organelle structures, improving photosynthesis, and reducing oxidative stress.

iii. Se inhibits fungal pathogen growth

Se is reported to inhibit mycelial growth of *S. sclerotiorum*, damage sclerotial ultrastructure, reduce the capacity of acid production, decrease superoxide dismutase and catalase activities, and increase the content of hydrogen peroxide and superoxide anion in mycelium, all of which result in the reduction of sclerotial formation in Oilseed rape (Cheng et al., 2019). Moreover, the study also revealed that the Se treatment increased the Se concentration in sclerotia, which inhibited sclerotial germination (Cheng et al., 2019). Regarding for *B. cinerea*, the selenite treatment at 24 mg L^{-1} significantly inhibited the spore germination of fungal pathogen

TABLE 1 Applications of Se treatments to study plant-pathogen interactions.

Treatment	Pathogens	Host	Disease	Reference
Na_2SeO_3 (0.052 – 4.0%) Na_2SeO_3 (0.5 – 40 mg L^{-1})	*Aspergillus flavus*	Brazil nut	Aspergillus flavus disease	(Ragab et al., 1986; Zohri et al., 1997; Li et al., 2003; Pacheco and Scussel, 2007)
Na_2SeO_3, Na_2SeO_4 (10 mg L^{-1}) Se-nanoparticles (100 mg L^{-1})	*Alternaria solani*	Tomato	Early blight of tomato	(Razak et al., 1991; Joshi et al., 2019)
Na_2SeO_3 (20 mg L^{-1})	*Fusarium oxysporum*	Tomato	*Fusarium* wilt	(Companioni et al., 2012)
Na_2SeO_3 (24 mg L^{-1}) Na_2SeO_4 (1 mg L^{-1})	*Botrytis cinerea*	Tomato	Gray mold disease	(Wu et al., 2015; Zhu et al., 2016)
Na_2SeO_3 (20 mg L^{-1})	*Penicillium expansum*	Apple	Blue mold rot	(Wu et al., 2014)
Na_2SeO_3 (5 mg L^{-1}) Na_2SeO_3 (0.1 mg kg^{-1}; 0.5 mg kg^{-1})	*Sclerotinia sclerotiorum*	Oilseed rape	Sclerotinia stem rot	(Cheng et al., 2019; Liu et al., 2019; Xu et al., 2020)
Na_2SeO_3, Na_2SeO_4, SeMet, $SeCys_2$ (20 mg L^{-1})	*Fusarium graminearum*	Wheat	Fusarium head blight	(Mao et al., 2020a; Mao et al., 2020b)

and the germ tube elongation in harvested tomato fruit (Wu et al., 2014; Wu et al., 2015). The membrane integrity, spore germination, germ tube elongation and mycelial spread of *P. expansum* were decreased significantly after the conidia were treated with Se of 20 mg L^{-1} for 9 h, and the inhibitory effect was positively related to the Se concentration in the growth medium (Wu et al., 2014). When spraying selenate on the leaves during fruit occurrence and development, Se can effectively control tomato gray mold *via* stimulating the antioxidant defense system of tomato plants (Zhu et al., 2016).

It has been reported that high levels of Se treatment led to the reduction of the proliferation and growth rate of *A. flavus*, and the decrease of the production of aflatoxin, which might be due to the toxic effects of Se on fungi (Pacheco and Scussel, 2007). The vegetative growth of *A. flavus* was inhibited with the increasing of the Se concentration, but the spores could not likely be damaged by selenite but only inhibited during germination (Zohri et al., 1997). In addition, high Se concentrated treatments led to the morphological distortion of fungal structure and deformities (Ragab et al., 1986; Li et al., 2003). Addition of different Se compounds to the toxin induction medium not only delays the growth of *F. graminearum* and reduces the diameter of colony, but also significantly inhibits the accumulation of deoxinivalenol (Mao et al., 2020b). Similarly, selenite has a certain inhibitory effect on *Fusarium oxysporum*, and the application of selenite

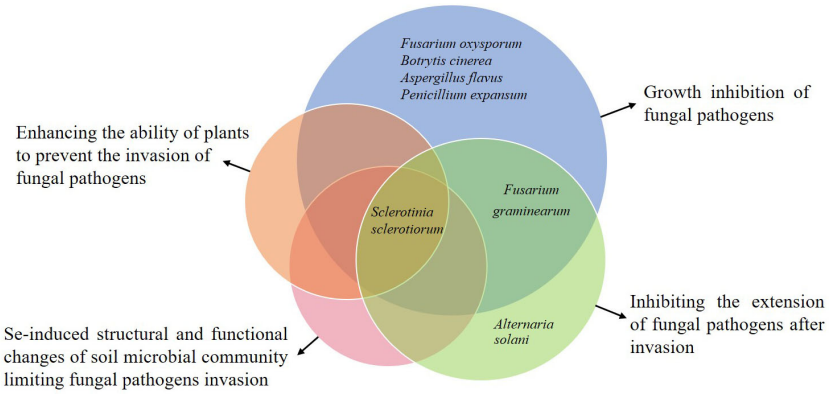

FIGURE 1
Various effects of Se on the specific fungal diseases observed in the previous studies. Each pie represents a specific type of impact. The pink, orange, blue, and green pies indicate the different impacts, including the Se-induced structural and functional changes of soil microbial community limiting fungal pathogens invasion, enhancing the ability of plants to prevent the invasion of fungal pathogens, growth inhibition of fungal pathogens, and inhibiting the extension of fungal pathogens after invasion, respectively. The overlap of blue and green pies shows that Se has interactive impacts on *Fusarium graminearum*, and the overlap of all pies shows the overall impact of Se on *Sclerotinia sclerotiorum*.

substantially reduces the number of wilted leaves per plant in susceptible tomato plantlets, and also results in wilt symptoms in the tomato plantlets (Companioni et al., 2012). As revealed above, Se can damage the cell structure of fungal pathogens and the plasma membrane of conidia, affect the osmotic regulation, reduce the vitality of pathogenic fungi, and finally inhibit mycelium growth.

iv. Se limits the extension of fungal pathogens after invasion

The extension of pathogens after invasion can be inhibited by Se through changes of cell tissue characteristics and physiological and biochemical reactions. The application of Se in soil can significantly increase the contents of tyrosine, tryptophan, pyroglutamic acid, histidine, glutamine, L-glutamic acid, aspartic acid and γ-aminobutyric acid in rape inoculated with *S. sclerotiorum* (Xu et al., 2020). Se (e.g. 0.1 mg kg^{-1} and/or 0.5 mg kg^{-1}) increased the activities of antioxidant enzymes such as catalase, polyphenol oxidase and peroxidase in plants. Particularly, Se leads to the up-regulation of defense genes including *CHI*, *ESD1*, *NPR1*, and *PDF1.2* in rapeseed leaves (Xu et al., 2020). Clearly, Se treatments are greatly beneficial for plants to defend against pathogens. Spraying organic Se solution (SeMet and $SeCys_2$) can inhibit the extension of *F. graminearum* in wheat ears and also reduce the percentage of diseased spikelets (Mao et al., 2020a). It was speculated that Se regulates the toxin production of *F. graminearum* by inhibiting the secretion of toxic substances, which was mediated by ATP-binding cassette transporter to reduce the accumulation of deoxinivalenol. The treatments with Se-nanoparticles (e.g. 10, 25, 50, and 100 mg kg^{-1}) could effectively inhibit the invasion and extension of *A. solanacearum* on pepper and tomato leaves pre-infected by *A. solanacearum* (Joshi et al., 2019). Se can stimulate plants to develop mechanistically important defense processes against pathogen, including the activation of defense genes and the production of secondary metabolites to mediate the host immunity and signal transduction regulation to resist pathogens (Xu et al., 2020).

Selenium-mediated plant resistance to insect pests

According to the response of plants to insect pests (Table 2 and Figure 2), the influence mechanisms of Se on plant resistance to insect pests are summarized as follows: (1) Se accumulated in plants can be metabolized into volatile compounds primarily DMSe and/or DMDSe as insect repellents, which negatively affect the ovipositing and feeding behaviors of insect pests; (2) The high concentration of Se accumulated in plants cause direct toxic effects on some pests, resulting in the increase of pest mortality, the decrease of reproduction rate, the inhibition of growth and development, and the shortening of adult life span.

i. Effects of Se on antixenosis

Traits that deter herbivores from feeding or oviposition (a phenomenon also referred to as antixenosis) can improve plant reproductive success by reducing the herbivore load of a focal plant while increasing herbivory on competitors (Erb, 2018). Often, antixenosis is rapid and conveniently determined, and it is sometimes more sensitive than performance as herbivores have potent sensory systems to choose between different food sources and oviposition sites (Reisenman et al., 2009).

The effects of Se on antixenosis have been reported in several insect pests. The Se-enriched diet acts as antifeedant for larvae of *S. exigua* and influences their selection of plants and feeding tissues or sites (Trumble et al., 1998; Vickerman and Trumble, 1999). *S. litura* is a polyphagous

TABLE 2 Applications of Se treatments to study pest-plant interactions.

Treatment	Insect pests	Effects	Reference
Na_2SeO_3 (0.125, 0.25, or 0.5%)	*Tenebrio molitor*	Antibiosis	(Hogan and Razniak, 1991)
Na_2SeO_3, Na_2SeO_4, SeMet, $SeCys_2$ (10, 30, 50, 70 mg kg^{-1})	*Spodoptera exigua*	Antixenosis and Antibiosis	(Trumble et al., 1998; Vickerman and Trumble, 1999; Vickerman et al., 2002)
Na_2SeO_4 (2 mg kg^{-1})	*Pieris rapae*	Antixenosis and Antibiosis	(Hanson et al., 2003)
Na_2SeO_4 (2, 40 μM)	*Acheta domesticus*	Antixenosis	(Freeman et al., 2007)
Na_2SeO_4 (10 mg kg^{-1})	*Myzus persicae*	Antixenosis and Antibiosis	(Hanson et al., 2010)
Na_2SeO_3 (11.9 mg kg^{-1}; 27.7 mg kg^{-1})	*Centroptilum triangulifer*	Antibiosis	(Conley et al., 2011)
Na_2SeO_3 (0.5, 0.75, 1, 2.5, 5 mg kg^{-1})	*Ostrinia furnacalis*	Antibiosis	(Han et al., 2017)
Na_2SeO_4 (6.5 ± 1.5 μM)	*Nilaparvata lugens*	Antibiosis	(Scheys et al., 2020)
Se-nanoparticles (25, 50, 75, 100 mg L^{-1})	*Spodoptera litura*	Antibiosis	(Arunthirumeni et al., 2022)

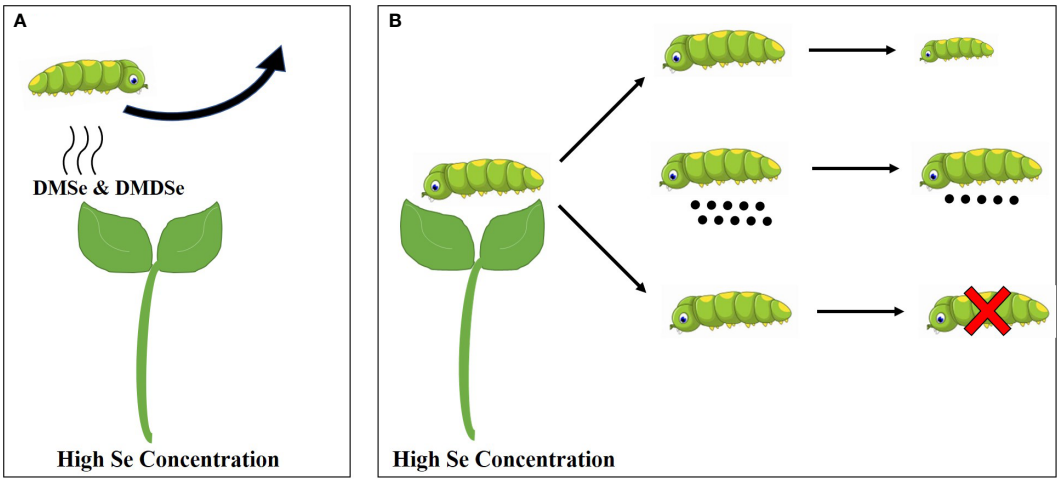

FIGURE 2
The defense model of plants against insect pests under high Se concentrated treatments. **(A)** High Se concentration and antixenosis and **(B)** High Se concentrations and antibiosis.

pest that causes extensive harm to cotton, peanut, tobacco, rice, corn, tea, broccoli, and cabbage (Senthil-Nathan, 2013; Lalitha et al., 2018). A study on *S. exigua* revealed that inorganic Se has antifeedant property to the older or over-matured larvae, but not with organic Se compounds (Vickerman et al., 2002). In particular, selenate is a deterrent agent against the plant's feeder, and organic Se compounds are less commonly used to avert pests, but biogenic volatile Se compounds (mainly DMSe or DMDSe) might act as the deterrent (Zayed, 1998). A choice feeding experiment demonstrated that crickets prefer eating plants with a lower Se content of 230 mg kg^{-1} rather than the plants with a higher Se content of 447 mg kg^{-1} (Freeman et al., 2007). Similarly, the choice feeding experiment using *P. rapae* showed that the larvae strongly preferred leaves without Se, and the feeding rates of leaves without Se was higher than that of Se-containing leaves (Hanson et al., 2003). In the experiment with mustard infected by *M. persicae*, the infection rate of plants without Se was significantly higher than that of plants with Se. After one week, the infection rate of plants without Se was close to 100% (Hanson et al., 2010). A recent study revealed that Se-nanoparticles exhibited a maximum antifeedant activity of 78.77%, and had toxic effects on larvae of *S. litura* (Arunthirumeni et al., 2022).

ii. Effects of Se on antibiosis of phytophagous insects

Antibiosis includes the adverse effect of the host-plant on the biology of the insects and their progeny (survival, development, and reproduction), and both chemical and morphological plant defenses mediate antibiosis (Padmaja, 2016). When plants absorb Se from the soil, Se can be transported from plants to insects through the food chain. Overall, for the possible harm or damages caused by phytophagous insects, the chemical protection mechanism of plants can be realized through the accumulation of Se, which can be explained using element defense hypothesis (Trumble and Sorensen, 2008).

It has been reported that the increasing concentrations of selenite or selenate solution significantly increased the time needed for development of *S. exigua* into the pupal and adult stages (Trumble et al., 1998). The time required to complete the larval stage was increased by 25%, and the time from egg to adult emergence was extended by 22-30% (Trumble et al., 1998). In a nonchoice feeding with mustard infected by *M. persicae*, aphid population growth was inversely correlated with the leaf Se concentrations (Hanson et al., 2010). It is worth noting that high levels of Se treatment could inhibit the development of aphid, but it also improved the resistance to virus in a different test (Shelby and Popham, 2007). Similarly, high Se concentrated treatments significantly affect the growth of *S. litura* larvae. When larvae were fed on treated plants with 25 mg L^{-1} selenite, the larvae weight was reduced by 40%. When the Se concentration was increased up to 50 mg L^{-1}, the growth of larvae was inhibited by 62%, and further, the growth of larvae was inhibited by 75% with the Se treatment of 100 mg L^{-1} (Popham et al., 2005). In addition, high Se concentrated treatments also inhibited the growth and development of *O. furnacalis*, which was characterized by reduced pupation and eclosion rates, decraesed pupae weights of both male and female, shortened longevity, and prolonged pupal duration (Han et al., 2017).

S. exigua larvae fed with Se-treated plant showed reduced body size and fecundity of adult moths from these larvae thereafter (Rothschild, 1969). It's reported that Se had negative effects on the reproduction of peach aphid (Hanson et al., 2010). Similarly, the Se concentration of 4.2 mg kg^{-1} decreased the fecundity of *C. triangulifer* (Conley et al., 2011). With the artificial diet containing 75 mg kg^{-1} of Se, *O. furnacalis* female had a lower courtship percentage and duration than the control, and the courtship peak time was delayed by 1 to 2 hours (Han et al., 2017). After larvae were fed with the artificial diet containing Se, it is possible that Se disrupts the biosynthesis and release of sex pheromones of *O. furnacalis*, which indeed affects its reproductive behavior (Han et al., 2017).

The mortality of terrestrial herbivores such as *T. molitor* due to Se toxicity could be significantly high (Hogan and Razniak, 1991; Trumble et al., 1998). When the Se concentration in leaves was 1.5

mg kg^{-1}, the growth of *M. persicae* population was decreased by 50%, and aphid began to die when the Se concentration was ≥ 10 mg kg^{-1} (Hanson et al., 2010). The newly hatched *P. rapae* larvae fed on plants with the Se concentration of 1300 mg kg^{-1} died within 9 days, and the 9-d-old caterpillars died at 2 days after exposure to plants with the Se concentration of 1600 mg kg^{-1} (Hanson et al., 2003). A recent study revealed that exposure of nymphs of *N. lugens* to 10.6 μM sodium selenite led to >80% mortality at 3 days after treatment, suggesting direct toxicity of selenium against this notorious insect pest (Scheys et al., 2020).

Conclusions and future research perspectives

Se plays an important role in plant growth and development, particularly enhancing the antioxidant capacity and increasing stress resistance of plants. The capacity of plants to inhibit pathogens and to resist diseases is related to maintaining the plant cell/organelle structure, reducing oxidative stress, inhibiting the mycelia growth and spore germination of pathogen, destructing the plasma membrane of conidia, and interfering pathogen's metabolism. In addition, the plant resistance to insect pests is also affected by selenium through deterring herbivorous insects from feeding or oviposition and leading to the death of early instars, reduced size or weight, prolonged periods of development of the immature stages, reduced adult longevity and fecundity, and the death in the prepupal or pupal stage. Based on the element defense theory, Se has been demonstrated to be effective in regulation and controlling of plant fungal diseases and insect pests. However, the specific effect may be related to the bioavailability, application methods and suitable sources (organic/inorganic/nanoparticles etc.).

The future research on Se and plant immunity needs to focus on mechanisms regarding the beneficial and toxic properties of different chemical forms of Se in plants. The practical exposure and dose ranges of Se on different fungal pathogens and insects also need to be well determined. The plant Se tolerance in relation to the biological characteristics of pathogens and phytophagous insects should be addressed when determining effects of different Se concentrations in different chemical forms. Previous studies primarily focused on fungal diseases, with only a few on bacterial and/or viral diseases. One might speculate that the effects of Se on bacteria and viruses would be similar to the effects of Se on fungi in plants, providing a research hypothesis that needs to be tested in future research. Due to potential biomagnification of Se through food chains, it may also be important to carefully monitor the Se accumulation in insects to ensure ecological safety during pest control particularly with Se-biofortified crop production.

Author contributions

QL and TL conceived the conceptual idea. QL wrote the initial draft of the paper. L-MX collected or retrieved research publications or inline the useful literature. LY, ZL, XC, JW and TL revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Impact of selenium biofortification on production characteristics of forages grown following standard management practices in Oregon

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Introduction: Low selenium (Se) concentrations in soils and plants pose a health risk for ruminants consuming locally-grown forages. Previous studies have shown that Se concentrations in forages can be increased using soil-applied selenate amendments. However, the effects of foliar selenate amendments applied with traditional nitrogen-phosphorus-potassium-sulfur (NPKS) fertilizers on forage yields, and nutrient contents, and agronomic efficiencies are unknown.

Methods: Using a split plot design, we determined the effects of springtime sodium selenate foliar amendment rates (0, 45, and 90 g Se ha^{-1}) and NPKS application (none, NPK for grasses/PK for alfalfa, and NPKS/PKS fertilization at amounts adapted to meet local forage and soil requirements) on forage growth and N, S, and Se concentrations, yields, and agronomic efficiencies. This 2-year study was conducted across Oregon on four representative forage fields: orchardgrass (*Dactylis glomerata* L.) in Terrebonne (central Oregon), grass-clover mixture in Roseburg (southwestern Oregon), and both grass mixture and alfalfa (*Medicago sativa* L.) fields in Union (eastern Oregon).

Results: Grasses grew poorly and were low in N content without NPK fertilization. Fertilization with NPK/PK promoted forage growth, increased forage N concentrations, and had to be co-applied with S when plant available S was low. Without Se amendment, forage Se concentrations were low and further decreased with NPKS/PKS fertilization. Selenate amendment linearly

increased forage Se concentration without adversely affecting forage yields, N and S concentrations, or N and S agronomic efficiencies.

Discussion: Importantly, S fertilization did not interfere with Se uptake in Se amended plots. In conclusion, co-application of NPKS/PKS fertilizers and foliar sodium selenate in springtime is an effective strategy to increase forage total Se concentrations, while maintaining optimal growth and quality of Oregon forages.

KEYWORDS

forage fertilization, grasses, legumes, nitrogen, selenium yield, selenium agronomic efficiency, sulfur

1 Introduction

Forage serves as an inexpensive, primary feed source for ruminant livestock operations (Schroeder, 2018). A challenge for livestock in many parts of the world, including Oregon, is that plant-available selenium (Se) concentrations are low (<0.1 mg Se/kg DM) in soils and locally produced forages (Oldfield, 2001). Selenium is an essential micronutrient for animals. Livestock consuming locally produced forages are susceptible to Se deficiency resulting in poor health and growth unless Se supplementation is provided. The health of livestock can be improved by feeding Se biofortified forages, a practice known as Se agronomic biofortification (Schiavon et al., 2020). Selenate amendment of pastures increases Se concentrations of forages, which, in turn, improves Se concentrations in forage-consuming cattle and sheep (Hall et al., 2009; Hall et al., 2011a).

A comparison across a limited number of studies varying the amount of Se applied per hectare suggests that there is linear increase in Se content of forage in response to Se dosage (Hall et al., 2009; Hall et al., 2013a; Wallace et al., 2017). The currently recommended Se application rates are 12.4-24.7 g Se ha^{-1}, as reviewed in (Brummer et al., 2014). However, we have previously shown health benefits in cattle and sheep fed supranutritional Se concentrations from forages grown on low-Se soil amended with 22.5, 45, and 90 g Se ha^{-1}. Thus, we are interested in Se dosages at concentrations that are higher than currently recommended by the US-FDA for preventing clinical Se deficiency (i.e., supranutritional dosages). At these higher concentrations we have observed improved production and fewer diseases without adverse health outcomes. We have evaluated supranutritional Se supplementation in Se-replete cattle and sheep throughout production stages, as well as during specific high-demand stages (e.g., during the backgrounding period before transport to the feedlot, and during the last 2–3 months of gestation) and have observed production and immune function improvements with both strategies in animals at the highest supplementation levels (Hall et al., 2011b; Stewart et al., 2012; Hall et al., 2013a; Hall et al., 2013b; Hugejiletu et al., 2013; Hall et al., 2014a; Hall et al., 2014b; Hall et al., 2017; Hall et al., 2020).

In contrast to animals, Se is not an essential nutrient for plants because plants utilize sulfur (S) rather than Se for their redox chemistry (reviewed in (White, 2018)). Plants do not have a SeCys-specific codon for peptide and protein synthesis, but rather both S and Se can incorporate interchangeably into the N-containing amino acids cysteine/SeCys and methionine/SeMet. In micro-mineral amounts (<100 mg Se kg^{-1} plant DM), Se may benefit plant health and growth because SeCys can more quickly and easily reverse protein oxidation and control redox processes than cysteine (Boldrin et al., 2016; White, 2018) thereby improving forage quality. In macro-mineral amounts (>100 mg kg^{-1} plant DM), Se is toxic for non-seleniferous plants, such as forage legumes and grasses (White, 2016).

Optimal forage production also depends on applying NPKS fertilizers (Moore et al., 2019). Fertilization with the macronutrients nitrogen (N) and S, or both, is an important part of forage nutrient management (Moore et al., 2019). Insufficient N results in low forage yield and quality, indicated by pale green colored leaves and low forage grass DM N concentrations (<2-2.5% plant DM) (Moore et al., 2019). Forage grasses depend on N fertilization, using ammonium and nitrate compounds for plant growth, protein synthesis, and tillering (Moore et al., 2019). In contrast, bacteria in the root region of legumes convert N gas from the air to ammonium-N compounds for plant growth and protein synthesis (Russelle et al., 1994). Nonetheless, grasses outcompete legumes in growth when fields are fertilized with N in the form of nitrate or ammonium-N compounds (Moore et al., 2019). To optimize forage yield and quality, N fertilization of grasses often requires co-application of S to satisfy the grasses' need for the S-containing amino acids cysteine and methionine for protein synthesis (Lancaster et al., 1971; Bolton et al., 1976; Aulakh, 2003; Moore et al., 2019). Sulfur fertilization using sulfate components provides plants with sulfate for root absorption. Forage S concentrations < 0.2% or a N:S ratio > 10 may indicate S deficiency (Moore et al., 2019; White et al., 2021).

Less research has focused on whether concurrent use of NPKS fertilizers affect Se biofortification (Li et al., 2007; Duncan et al., 2017). There are concerns that S fertilization may exacerbate forage Se deficiencies, because selenate and sulfate compete for the same root membrane transporter, the expression of which is increased during S depletion (Pratley and McFarlane, 1974; Lauchli, 1993; Gupta and Macleod, 1994; White et al., 2004; Li et al., 2008; Luo et al., 2019; Deng et al., 2021). However, foliar selenate amendment

may prevent the competition of Se and sulfur for root absorption (Ros et al., 2016; Ramkissoon et al., 2019; Schiavon et al., 2022).

Our objective is to make application of foliar selenate amendment a standard nutrient management practice for Oregon's Se-deficient pastures. To achieve our objective, we investigated over two years the effects of springtime sodium selenate foliar application (0, 45, and 90 g Se ha^{-1}) and nitrogen, phosphorus, potassium (NPK) fertilization, alone or in combination with S (NPKS), on forages grown throughout Oregon. We previously reported on the effect of selenate amendment and NPKS fertilization on forage Se concentrations, as well as Se species composition (Wang et al., 2021). In the current study, we focus on the effects of selenate amendment and NPKS fertilization on total forage biomass (indicative of forage growth), nutrient concentrations (indicative of forage and feed quality), nutrient yields (indicative of nutrient uptake), and nutrient agronomic efficiencies (indicative of Se amendment or fertilizer N and S use). We hypothesized, given the much lower amounts of selenate applied (*vs.* N, P, K and S), that application of standard NPKS fertilizers to Oregon's Se-deficient pastures should not interfere with Se yields or Se agronomic efficiencies.

2 Materials and methods

2.1 Experimental design and agronomic field management

The study was conducted from 2017 to 2019 using four irrigated hayfields at three different climatic sites, Roseburg (43.2°N, 123.3°W, and 161 m asl.) in southwestern Oregon, Terrebonne (44.4°N, 121.2°W, and 851 m asl.) in central Oregon, and Union (45.2°N, 117.9°W, and 853 m asl.) in eastern Oregon (Figure 1). The average growing seasons are much shorter in central (80 days) and eastern (119 days) Oregon compared with southwestern (182 days) Oregon. We collected rainfall and temperature data during the study period (May 2017 to May 2019): average rainfall and temperatures were higher in southwestern Oregon (Roseburg: 77 mm mo^{-1} and 11.4°C) compared with central and eastern Oregon (Terrebonne: 20 mm mo^{-1} and 8.3°C; Union: 40 mm mo^{-1} and 8.1°C). Whereas maximal monthly temperatures in summer were similar across the three sites (Roseburg: 34°C; Terrebonne: 32°C; Union: 33°C), average monthly temperatures in winter were milder in southwestern Oregon (Roseburg: 3°C) compared with central and eastern Oregon (-2°C). Rainfall was seasonal with nearly no precipitation (<1 mm mo^{-1}) in July and August, requiring irrigation during the summer to provide plants with sufficient water to off-set evapotranspiration. In central and southwestern Oregon, the growing season rainfall was on average in 2017, but unusually dry in 2018 (Terrebonne: 124 mm in 2017 and 15 mm in 2018; Roseburg: 60 mm in 2017 and 26 mm in 2018), with no differences in eastern Oregon (Union: 92 mm in 2017 and 91 mm in 2018).

The soil types were loam in Roseburg (11.0% clay, 62.8% sand, and 26.2% silt), sandy loam in Terrebonne (9.4% clay, 59.8% sand, and 30.8% silt), and silt loam in Union (22.5% clay, 9.5% sand, and 68.0% silt) (Table 1). The soil organic matter content was low in central and southwestern Oregon (Roseburg: 1%; Terrebonne: 1.7%) and high in Union (4.7-6.6% in the alfalfa field and 4.9-5.5% in the grass field). The cation exchange capacity (CEC) was higher in Union (alfalfa field: 36.2-36.7 mEq 100 g^{-1}; grass field:

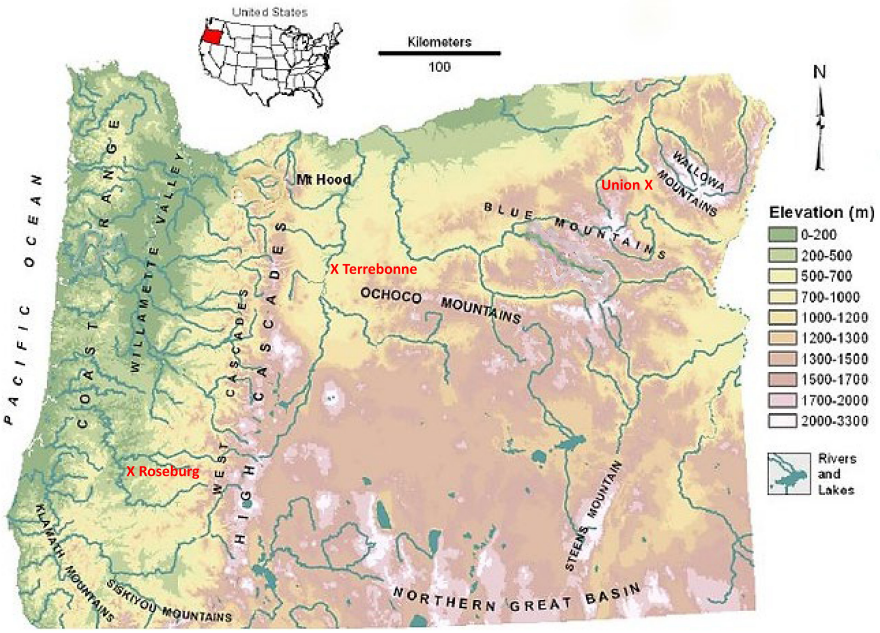

FIGURE 1
Map showing locations of forage sites at Union (eastern Oregon), Terrebonne (central Oregon), and Roseburg (western Oregon). Adapted from Leibowitz et al. (2014).

38.0-41.1 mEq 100 g^{-1}) compared with Terrebonne (13.8-17.0 mEq 100 g^{-1}) and Roseburg (18.0 mEq 100 g^{-1}). The soil pH values were acidic in Roseburg (pH: 5.6) and Terrebonne (pH: 5.5-5.7) and close to neutral in Union (grass field pH: 6.5-6.6; alfalfa field pH: 7.0-7.2). The soluble salt (SS) content of the soil was low at all three sites: Roseburg (0.1 mmhos cm^{-1}), Terrebonne (0.1-0.3 mmhos cm^{-1}), and Union (both 0.3 mmhos cm^{-1}). The total soil Se concentration was also low at all three sites: Roseburg (0.18 mg Se kg^{-1} DM), Terrebonne (0.12 mg Se kg^{-1} DM), and Union (0.15 mg Se kg^{-1} DM).

The diverse climatic and edaphic conditions impact the forage types that can be profitably grown at each site. The four forage fields were alfalfa (*Medicago sativa* L.) in Union; orchardgrass (*Dactylis glomeruata* L.) in Terrebonne; a grass mixture [primarily tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh. formerly *Festuca arundinacea* (Schreb.) and orchardgrass] in Union; and a 50:50 grass-clover mixture [dominated with tall fescue, orchardgrass, perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.), and subterranean clover (*Trifolium subterraneum* L.)] in Roseburg.

At each location, the experiment was laid out as a split-plot design with three replications, with selenate application rate as whole plot and NPKS fertilization protocol as split plot (Figure 2). The plot dimensions were 6.0 m × 1.5 m in Roseburg, 4.5 m × 1.5 m in Terrebonne, and 6.1 m × 2.4 m in Union, which was based on field location and research machinery availability. Sodium selenate (RETORTE Ulrich Scharrer GmbH, Röthenbach, Germany) was applied at rates of 0, 45, or 90 g Se ha^{-1} to the same field whole plots on May 5, 2017 and May 16, 2018 in Roseburg; April 21, 2017 and May 4, 2018 in Terrebonne; and May 2, 2017 and 2018 in Union (Table 2). Plants were 5 to 10 cm high and in the tillering phase of the vegetative stage with leaves covering most of the soil. First, sodium selenate was dissolved in water (500 mL of water per plot). To uniformly cover the entire treatment area, we calibrated prior to application a back-pack sprayer with a time/speed calibration method. Sufficient water was added so that the treatment could be applied in a consistent manner using a spray pressure and walking speed that was easily maintained by the applicator. The aqueous sodium selenate solution was applied with a backpack sprayer fitted with a precision nozzle to deliver the specified application rate.

Fertilization of (N)PKS included 0, (N)PK, and (N)PK plus S. The same agronomic principles were applied to meet forage species requirements for (N)PK and (N)PKS at each location (Gardner et al., 1981; Gardner et al., 2000; Koenig et al., 2009; Horneck et al., 2011; Miller et al., 2013; Moore et al., 2019). Based on soil analyses (Table 1) and production potential for each site, recommended fertilizer amounts of (N)PK and S were applied using appropriate combinations of gypsum, ammonium sulfate, ammonium phosphate, and urea. Soil analyses were performed by Ag Source Laboratories (Umatilla, OR) at the beginning of each growing season in 2017 and 2018 (Table 2). Soils were sampled from 0 to 15 cm in Roseburg, and 0 to 30 cm in Terrebonne and Union. At Roseburg, fertilizer included 0 (none), NPK (40 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1}, and 100 kg K_2O ha^{-1}), and NPKS (NPK plus 34 kg S ha^{-1}). Fertilizer was applied one day before Se application each year. In addition, urea (50 kg N ha^{-1}) was applied to the treated plots after the first and second cuts in 2017, and after the first cut in 2018. At Terrebonne, fertilizer included 0 (none), NPK (134.5 kg N ha^{-1}, 33.6 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1}), and NPKS (NPK plus 33.6 kg S ha^{-1}). Fertilizer was applied on the day of Se application in 2017 and ten days before Se application in 2018. NutriSphere-N®-coated urea was applied after the first cut (134.5 kg N ha^{-1}) and after the second cut (67.25 kg N ha^{-1}). At Union, fertilizer included 0 (none), NPK (72.0 kg N ha^{-1}, 34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and NPKS (NPK plus 12.0 kg S ha^{-1}). Fertilizer was applied three days before Se application in 2017 and on the day of Se application in 2018. Urea (72.0 kg N ha^{-1}) was applied to grasses after the first cut each year. For alfalfa, N was not applied.

Forages were harvested based on the recommended maturity stage (Collins et al., 2018); alfalfa was harvested at 10% bloom stage, and grass-dominated forages were harvested at early-anthesis stage on first cut and vegetative stage on the following cuts. At Roseburg, plots were harvested three times (25, 65, and 114 days post Se application) in year one, and twice (63 and 128 days post Se application) during year two, the latter due to a dry spring with slow forage growth (Table 2). Plots were harvested by a 0.9 m wide cycle-bar mower with a cutting height of 5 cm. At Terrebonne, plots were harvested 54, 104, and 157 days post Se application in year one and 32, 88, and 128 days post Se application in year two. Plots were harvested by a 1.1 m wide small plot sickle bar mower with a cutting height of 10 cm. At Union, alfalfa plots were harvested three times (42, 93, and 163 days post Se application) during year one and three times (43, 90, and 147 days post Se application) during year two, and grass plots were harvested twice (42 and 166 days post Se application) during year one and twice (23 and 147 days post Se application) during year two. Plots were harvested by a 0.9 m wide flail type harvester with a cutting height of 7.6 cm.

At the beginning of the new growing season, residual growth (i.e., regrowth before Se application) was collected on March 29, 2018 and March 30, 2019 in Roseburg. At Union, residual growth was clipped on April 30 and May 1, 2018 for alfalfa and grasses, respectively, and May 14, 2019. At Terrebonne, residual growth was clipped on April 15, 2018. In 2019, the integrity of the plots was not discernable to sample residual growth. The fields at Terrebonne and Union were utilized from October until mid-April as beef cattle pastures and the plots in Roseburg were mowed and the forage removed in the second half of October and late March. At the beginning of a new growing season, manure piles, if present, were removed.

2.2 Laboratory analytical methods

Representative forage grab samples (20 per plot in Roseburg and Union) were collected at each harvest time. We did not adjust for plant species differences. Forage samples were dried within hours of collection for at least 48 hours or until they reached a constant dry weight at 65°C. Samples were then ground with a Wiley mill with a 1.0 mm screen. At Terrebonne, 4 representative grab samples from each plot totaling 300-450 grams of forage were placed into Super 12 U-line paper bags and weighed within 10

TABLE 1 Soil analysis for forage sites at Union (eastern Oregon), Terrebonne (central Oregon), and Roseburg (western Oregon)[1].

Site/ Forage/ Date	Plot	pH	pH Buffered	OM[2]	SS	CEC	P	K	Mg	Ca	Na	NO_3	NH_4	S
				g $100g^{-1}$	dS m^{-1}	Cmol(+) kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Union/Alfalfa	Soil type: silt loam (22.5% clay, 9.5% sand, and 68.0% silt)						Olsen[3]							
Guidelines[4,5,6]		6-8.2	NA[1]	NA	<1.0	NA	20	200	NA	NA	<10.0	NA	NA	15
19.04.2017	Baseline	7.0	7.0	4.2	0.2	31.3	23	178	4	11.5	0.3	14.4	18.25	11
16.04.2018	None	7.0	6.9	4.7	0.3	36.7	26	208	4.55	13.4	0.36	22	19	21.6
16.04.2018	PK	7.1	7.1	6.6	0.3	36.2	25	191	4.55	13.15	0.33	28.5	22.5	23.2
16.04.2018	PKS	7.2	7.1	4.7	0.3	36.3	21	156	4.65	13.1	0.35	22	21	12.9
Union/ Grasses	Soil type: silt loam (22.5% clay, 9.5% sand, and 68.0% silt)						Olsen[3]							
Guidelines[4,7]		5.6	NA	NA	<1.0	NA	10	150	NA	NA	<10.0	7	NA	NA
19.04.2017	Baseline	6.3	6.6	5.6	0.2	40.3	32	205	5	12	0.3	18.5	22	16
16.04.2018	None	6.6	6.6	4.9	0.3	38.0	29	231	5.45	13.05	0.33	11.5	18.5	16.7
16.04.2018	NPK	6.6	6.7	4.9	0.3	38.6	22	207	5.7	13.2	0.32	8.5	16.5	15.2
16.04.2018	NPKS	6.5	6.8	5.5	0.3	41.1	29	193	5.5	13.15	0.31	10.5	27	14.7
Terrebonne Orchard Grass	Soil type: sandy loam (9.4% clay, 59.8% sand, and 30.8% silt)						Olsen[3]							
Guidelines[4,7]		5.6	NA	NA	<1.0	NA	10	150	NA	NA	<10.0	7	NA	NA
6.04.2017	Baseline	5.6	6.7	1.7	0.1	13.8	23	116	1.5	3.1	0.26	2.5	4	9.7
24.04.2018	None	5.7	6.6	ND	0.3	17.0	27	147	1.85	3.75	0.38	14.5	25.5	14.5
24.04.2018	NPK	5.6	6.6	ND	0.3	16.4	27	123	1.75	3.6	0.35	9	17	11.9
24.04.2018	NPKS	5.6	6.9	ND	0.3	13.9	29	120	1.8	3.6	0.36	10	21.5	13.6
Roseburg Grass-Clover	Soil type: loam (11.0% clay, 62.8% sand, and 26.2% silt)						Bray[3]							
Guidelines[4,8]		5.5	NA	>2.0	<1.0	NA	30	200	0.8	5.0	<10.0	NA	NA	NA
12.05.2017	Baseline	5.6	6.8	ND	0.1	18.0	43	186	2.2	4.95	0.13	ND	ND	ND
02.04.2018	None	6.1	6.5	ND	0.2	20.2	9	159	2.15	4.65	0.17	ND	ND	ND
02.04.2018	NPK	6.1	6.7	ND	0.1	18.4	8	161	2.2	4.7	0.16	ND	ND	ND
02.04.2018	NPKS	6.0	6.8	ND	0.2	17.4	8	138	2.2	4.75	0.17	ND	ND	ND

[1]Soil samples were taken 0 to 15.24 cm in Roseburg and 0 to 30.48 cm in Union and Terrebonne. Samples were analyzed by AgSource Laboratories in Umatilla (Oregon).
[2]OM, organic matter; SS, soluble salts (measure of the amount of nutrients in solution in the form of electric conductivity dS/m); CEC, cation exchange capacity (measure of the amount of cations a soil can adsorb by cation exchange, usually expressed as cmol(+) kg^{-1}); P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; NO_3, nitrate; NH_4, ammonium; S, sulfate-sulfur; NA, not available; ND, not determined.
[3]Olsen and Bray indicate methods for phosphorus determination. The Bray test is unreliable at soil pH > 7.45. Olsen value = 3.5 + (0.42 x Bray value). The Olsen sodium bicarbonate extraction method is used for soils east of the Cascade Mountain Range (Union and Terrebonne) and the Bray P1 extraction method is used for soils west of the Cascade Mountain Range (Roseburg, OR). Soil, Plant and Water Reference Methods for the Western Region (Miller et al., 2013).
[4]Soil Test Interpretation Guide (Horneck et al., 2011).
[5]Nutrient Management Guide for Dryland and Irrigated Alfalfa in the Inland Northwest (Koenig et al., 2009).
[6]Alfalfa (eastern Oregon – east of the Cascades) (Gardner et al., 1981).
[7]Irrigated clover-grass pastures: eastern Oregon – east of Cascades (Gardner et al., 2000).
[8]Nutrient Management Guide for Western Oregon and Washington Pastures (Moore et al., 2019).

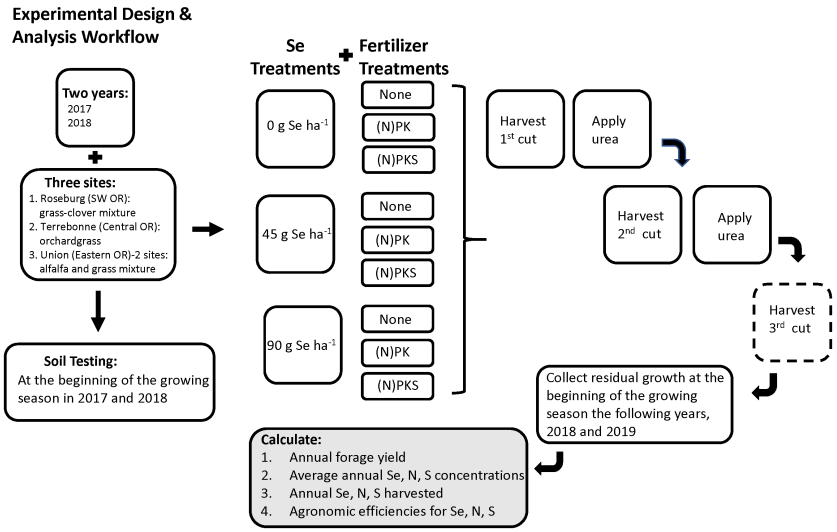

FIGURE 2
Experimental design and analysis workflow for soil sample collection, Se and fertilizer treatments, and forage sample cuttings taken at each of the testing sites in Oregon: Union (eastern Oregon), Terrebonne (central Oregon), and Roseburg (western Oregon). Each Se by fertilizer treatment combination was performed in triplicate at each forage species site. The third cut harvest was collected when possible.

TABLE 2 Timeline for soil sample collection, Se and fertilizer treatments, and forage sample cuttings taken at each of the testing sites in Oregon: Union (eastern Oregon), Terrebonne (central Oregon), and Roseburg (western Oregon).

	Roseburg	Terrebonne	Union	Union
	Grass-clover mixture	Orchard grass	Alfalfa	Grass mixture
Baseline soil samples collected	May 4, 2017	April 6, 2017	April 15, 2017	April 15, 2017
Fertilizer treatments, 2017	May 4, 2017	April 21, 2017	April 29, 2017	April 29, 2017
Se treatments, 2017	May 5, 2017	April 21, 2017	May 2, 2017	May 2, 2017
Harvest 1st cut, 2017	May 30, 2017	June 14, 2017	June 13, 2017	June 13, 2017
Apply urea, 2017	May 31, 2017	June 22, 2017	NA	July 26, 2017
Harvest 2nd cut, 2017	July 9, 2017	August 3, 2017	August 3, 2017	October 15, 2017
Apply urea, 2017	July 10, 2017	August 9, 2017	NA	NA
Harvest 3rd cut, 2017	September 14, 2017	September 25, 2017	October 12, 2017	NA
Collect residual growth, 2018	March 29, 2018	April 24, 2018	April 30, 2018	May 1, 2018
Soil samples collected	March 28, 2018	April 24, 2018	April 12, 2018	April 12, 2018
Fertilizer treatments, 2018	May 15, 2018	April 24, 2018	May 2, 2018	May 2, 2018
Se treatments, 2018	May 16, 2018	May 4, 2018	May 2, 2018	May 2, 2018
Harvest 1st cut, 2018	July 17, 2018	June 5, 2018	June 14, 2018	May 25, 2018
Apply urea, 2018	July 18, 2018	June 13, 2018	NA	June 1, 2018
Harvest 2nd cut, 2018	September 22, 2018	July 31, 2018	July 31, 2018	September 26, 2018
Apply urea, 2018	September 23, 2018	August 6, 2108	NA	NA
Harvest 3rd cut, 2018	NA	September 7, 2108	September 26, 2018	NA
Collect residual growth, 2019	March 2019	NA	May 14, 2019	May 14, 2019

NA, None applied or no harvest; Not available.

minutes of collection with a portable Scout electronic scale to determine moist-sample weights in the field. The samples were then transported to a forage dryer and dried at 65°C until there was no longer any change in weight (approximately 3 days). Samples were then reweighed to calculate percent dry matter. Annual forage yield (kg forage DM ha^{-1}) was determined by multiplying forage yield on a wet basis (kg forage ha^{-1}) by percent dry matter.

The ground forage samples (approximately 50 g) were sent to the Utah Veterinary Diagnostic Laboratory (Logan, UT) to measure total Se concentration. As previously described (Davis et al., 2012), forage samples were prepared for Se analysis and Se was determined using inductively coupled argon plasma emission spectroscopy (ICP-MS; ELAN 6000, Perkin Elmer, Shelton, CT). Quantification of Se was performed by the standard addition method, using a 4-point standard curve. A quality-control sample (in similar matrix) was analyzed after every 5 samples, and analysis was considered acceptable if the Se concentration of the quality-control sample fell within ± 5% of the standard/reference value.

Ground forage samples (approximately 50 g) were sent to the Soil Health Laboratory at Oregon State University. Total carbon (C), nitrogen (N), and sulfate-sulfur (S) concentrations were determined by dry combustion using an Elementar vario macro cube (Elementar, Hanau, Germany). Forage samples were wrapped in aluminum foil and dropped into the analyzer through a blank-free helium-purged ball valve, and oxygen was injected over the sample at 1150°C. Separation of combustion gases was performed using a thermal desorption purge and trap chromatographic method (Bernius et al., 2014). The combustion gas components CO_2 and SO_2 were adsorbed onto two specific columns. Nitrogen flowed directly to a thermal conductivity detector. Based on the detector reading, gas components were released sequentially from their individual adsorption/desorption columns. Total time for analysis for carbon, N, and S was 10 minutes/sample.

2.3 Statistical analysis

The experimental design was a split-plot design with repeated measures at four forage-site combinations. Each split plot was replicated in triplicate. The whole plot was selenate amendment rate and the split-plot was fertilizer type. There were three replicates for each combination of amendment rate and fertilizer type.

Agronomic efficiencies were calculated as follows. For Se agronomic efficiency (%) = [(Se yield with selenate amendment – Se yield without selenate amendment)/amount of selenate amended] x 100. For N agronomic efficiency (%) = [(N yield with NPK(S) fertilization – N yield without NPK fertilization)/amount of N applied] x 100. For S agronomic efficiency (%) = [(S yield with (N)PKS application – S yield with (N)PK application)/amount of S applied] x 100.

The data were analysed as intention-to-treat analysis in PROC MIXED in SAS version 9.4 (SAS, 2009). Fixed effects in the model were foliar springtime selenate amendment rate (0, 45, or 90 g Se ha^{-1}), the fertilizer type (none, (N)PK, or (N)PKS), and their interaction. The random effect was replicate (1, 2, or 3). Data are shown in Tables 3–5 as least-squared means (LSM) and a pooled standard error of differences (SED). To determine LSM and SED, the statistical model was run for each forage/site × year combination separately. The *P*-values in the tables refer to those of the fixed effects in the model.

Using ESTIMATE statements, the linear effect of selenate amendment rate was calculated by comparing results of 90 g Se ha^{-1} amended plots with those of 0 g Se ha^{-1} amended plots; the non-linear or lack of linearity effect of selenate amendment rate was calculated by comparing results for 45 g Se ha^{-1} amended plots with those of 0 and 90 g Se ha^{-1} amended plots combined; the effect of (N)PK-fertilization was calculated by comparing results for (N)PK-fertilized with non-fertilized plots; and the effect of S-fertilization was calculated by comparing results for (N)PKS-fertilized with (N)PK-fertilized plots. All contrasts were orthogonal. The *P*-values in the text refer to those calculated by the ESTIMATE statements. PROC CORR was used to calculate Pearson's correlation coefficients. All statistical tests were two-sided. Statistical significance was declared at $p \leq 0.05$ and a statistical trend was declared at $0.05 < p \leq 0.10$.

3 Results

3.1 Response of forages to different selenate application rates

Foliar springtime selenate amendment at 45 and 90 g Se ha^{-1} did not affect annual forage yields (kg forage DM ha^{-1}) in Oregon in 2017 and 2018 (all main effects $P > 0.30$; Table 3; Figure 3). Soil Se concentrations were low at all three sites: Roseburg (0.18 mg Se kg^{-1} soil), Terrebonne (0.12 mg Se kg^{-1} soil), and Union (0.15 mg Se kg^{-1} soil). Plant Se concentrations without selenate amendment were lower than soil Se concentrations: Roseburg (0.08-0.10 mg Se kg^{-1} forage DM), Terrebonne (0.04-0.11 mg Se kg^{-1} forage), and Union (0.07-0.20 mg Se kg^{-1} forage with lower concentrations in 2017 than in 2018). Without Se-amendment, alfalfa had higher Se concentrations (2018) and yields (2017 and 2018) than grasses at Union. After Se-amendment, alfalfa had lower Se concentrations (2018) than grasses.

Selenate amendment at 45 and 90 g Se ha^{-1} increased average Se concentrations (mg Se kg^{-1} forage) and annual Se yield (g Se ha^{-1}) of the harvested forage (Table 3; Figure 4). The effect was significant at both amendment rates for all combinations of forage species, locations and years (all main effects $P < 0.004$). Selenate amendment linearly increased forage Se concentrations (linearity: all $P < 0.004$; lack of linearity: all $P > 0.31$) and annual Se yield (linearity: all $P < 0.002$; lack of linearity: all $P > 0.42$). Forage Se concentrations were highly correlated to annual Se yield ($r = +0.70$; $P < 0.0001$), but not to yields and concentrations of N or S (all $P > 0.03$). Among Se-amended plots, grasses at Union in 2018 had the highest Se concentration, whereas grass-clover at Roseburg in 2018 had the lowest.

Selenium agronomic efficiency (%) after selenate amendment, calculated by the formula [(Se yield with selenate amendment – Se yield without selenate amendment)/amount of selenate amended] x 100, was strongly correlated to annual forage yield ($r = +0.81$; $P < 0.0001$) and was not affected by selenate amendment rate (all $P > 0.47$). Alfalfa at Union and NPKS-fertilized orchard grass at

TABLE 3 Effect of springtime sodium-selenate foliar application rate (0, 45, and 90 g Se ha^{-1}) and nitrogen-phosphorus-potassium-sulfur fertilization (none, (N)PK, or (N)PKS) on forage yield (kg ha^{-1} DM) and forage Se concentration, yield, and agronomic efficiency in forages across Oregon[1].

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
Year & Forage Type	**Annual Forage Yield (kg forage DM ha^{-1})**												
2017													
Alfalfa[2]	10505	10671	11107	12603	12087	12782	11999	12570	11743	546	0.91	<0.0001	0.18
Grasses[2]	2090	3548	1876	3981	3608	3592	4857	4582	3596	1027	0.55	0.005	0.40
Orchard Grass[3]	3491	4354	3479	10507	10155	9711	11838	11659	11769	773	0.80	<0.0001	0.53
Grass-Clover[4]	3360	3253	3936	5416	5369	5189	5233	5435	5462	453	0.80	<0.0001	0.53
2018													
Alfalfa	12197	11663	11395	14092	13953	15118	14888	13379	13869	771	0.31	0.0001	0.35
Grasses	1472	3807	2203	5004	4712	4459	4980	4712	3978	1483	0.87	0.01	0.30
Orchard Grass	4028	4894	4158	9718	8947	10561	14881	14436	14468	949	0.93	<0.0001	0.096
Grass-Clover	6493	5799	6645	7553	7540	8111	7768	8493	8284	1143	0.89	0.002	0.70
	Average Annual Se Concentration (mg Se kg^{-1} forage DM)												
2017													
Alfalfa	0.10^{c}	1.46^{b}	2.93^{a}	0.13^{c}	1.44^{b}	2.76^{a}	0.07^{c}	1.22^{b}	2.71^{a}	0.28	<0.0001	0.45	0.92
Grasses	0.11^{c}	1.51^{b}	2.61^{a}	0.09^{c}	1.51^{b}	2.58^{a}	0.07^{c}	1.32^{b}	3.21^{a}	0.25	<0.0001	0.57	0.16
Orchard Grass	0.08^{c}	1.13^{b}	2.88^{a}	0.07^{c}	1.02^{b}	2.11^{a}	0.08^{c}	1.13^{b}	2.41^{a}	0.26	<0.0001	0.10	0.26
Grass-Clover	0.08^{c}	1.30^{b}	2.58^{a}	0.09^{c}	1.15^{b}	2.52^{a}	0.09^{c}	1.19^{b}	2.48^{a}	0.46	0.003	0.84	0.99
2018													
Alfalfa	0.18^{c}	1.35^{b}	1.98^{a}	0.20^{c}	1.06^{b}	2.00^{a}	0.16^{c}	1.03^{b}	1.81^{a}	0.25	<0.0001	0.32	0.69
Grasses	0.13^{c}	1.50^{b}	3.02^{a}	0.12^{c}	1.69^{b}	3.60^{a}	0.14^{c}	1.45^{b}	3.81^{a}	0.46	<0.0001	0.52	0.64
Orchard Grass	0.10^{c}	1.25^{b}	2.65^{a}	0.11^{c}	0.83^{b}	2.03^{a}	0.04^{c}	1.18^{b}	2.32^{a}	0.24	<0.0001	0.09	0.44
Grass-Clover	0.09^{c}	0.86^{b}	1.30^{a}	0.09^{c}	0.67^{b}	1.29^{a}	0.10^{c}	0.72^{b}	1.18^{a}	0.11	<0.0001	0.39	0.63
	Annual Se Harvested (g Se ha^{-1})												
2017													
Alfalfa	1.07^{c}	15.55^{b}	32.37^{a}	1.60^{c}	17.44^{b}	35.45^{a}	0.89^{c}	15.31^{b}	31.73^{a}	3.39	<0.0001	0.35	0.94
Grasses	0.21^{b}	5.59^{a}	4.82^{a}	0.37^{b}	5.43^{a}	9.24^{a}	0.36^{c}	6.09^{b}	11.57^{a}	2.12	0.009	0.045	0.053
Orchard Grass	0.29^{b}	5.26^{ab}	10.03^{a}	0.70^{c}	10.60^{b}	20.49^{a}	0.93^{c}	13.33^{b}	28.32^{a}	2.84	0.0003	0.0001	0.005
Grass-Clover	0.28^{b}	4.30^{b}	10.36^{a}	0.45^{c}	6.16^{b}	12.86^{a}	0.44^{c}	6.30^{b}	13.60^{a}	2.26	0.004	0.02	0.33
2018													
Alfalfa	2.23^{c}	15.76^{b}	22.72^{a}	2.79^{c}	14.83^{b}	29.99^{a}	2.31^{c}	14.03^{b}	25.02^{a}	3.54	0.0004	0.31	0.32
Grasses	0.18^{b}	3.92^{ab}	6.65^{a}	0.61^{c}	7.15^{b}	15.63^{a}	0.71^{c}	6.64^{b}	15.12^{a}	2.53	0.0004	0.03	0.20
Orchard Grass	0.41^{b}	6.16^{ab}	10.93^{a}	1.09^{b}	7.49^{b}	21.77^{a}	0.55^{c}	17.07^{b}	33.51^{a}	3.26	0.0004	<0.0001	0.001
Grass-Clover	0.61^{c}	5.01^{b}	8.66^{a}	0.72^{c}	5.07^{b}	10.41^{a}	0.75^{c}	6.22^{b}	9.72^{a}	1.15	0.0004	0.09	0.17
	Se Agronomic Efficiency (%)[5]												
2017													
Alfalfa	Ref.[6]	32.17	34.77	Ref.	35.21	37.61	Ref.	32.04	34.61	5.20	0.55	0.56	0.99
Grasses	Ref.	11.96	5.12	Ref.	11.24	9.86	Ref.	12.74	12.46	4.46	0.47	0.27	0.36

(Continued)

TABLE 3 Continued

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
Orchard Grass	Ref.	11.06	10.83	Ref.	22.01	22.00	Ref.	27.55	30.43	6.15	0.85	0.003	0.89
Grass-Clover	Ref.	8.93	11.19	Ref.	12.69	13.78	Ref.	13.01	14.61	3.71	0.66	0.02	0.88
2018													
Alfalfa	Ref.	30.06	22.76	Ref.	26.76	30.22	Ref.	26.05	25.23	7.67	0.83	0.66	0.28
Grasses	Ref.	8.32	7.18	Ref.	14.52	16.68	Ref.	13.16	16.00	5.05	0.70	0.10	0.83
Orchard Grass	Ref.	12.79	11.69	Ref.	14.22	22.98	Ref.	36.70	36.63	6.09	0.63	0.0002	0.30
Grass-Clover	Ref.	9.78	8.93	Ref.	9.68	10.76	Ref.	12.17	9.96	2.64	0.79	0.31	0.33

[1]Data are shown as least-squared means (LSMs) of sodium selenate application rates within fertilization types with a pooled standard error of difference (SED) for each year. Forages were harvested 2 times (grasses both years and grass-clover in 2018) or 3 times (alfalfa and orchard grass both years and grass-clover in 2017) per year. LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with different lower-case superscripts are significantly different from each other (P≤0.05). LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with the same lower-case superscripts or no superscripts are not significantly different from each other (P≥0.05).

[2]For alfalfa and grasses at Union (eastern Oregon), sodium selenate (RETORTE Ulrich Scharrer GmbH, Röthenbach, Germany) was applied May 2, 2017 and 2018. Fertilizer was applied three days before selenate application in 2017 and on the day of selenate application in 2018 and included for alfalfa 0 (none), PK (34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and PKS (PK plus 12.0 kg S ha^{-1}) and for grasses 0 (none), NPK (72.0 kg N ha^{-1}, 34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and NPKS (NPK plus 12.0 kg S ha^{-1}). In addition, urea (72.0 kg N ha^{-1}) was applied after the first grass cut.

[3]For orchard grass at Terrebonne (central Oregon), sodium selenate was applied April 21, 2017 and May 4, 2018. Fertilizer was applied on the day of selenate application in 2017 and ten days before selenate application in 2018 and included 0 (none), NPK (134.5 kg N ha^{-1}, 33.6 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1}), and NPKS (NPK plus 33.6 kg S ha^{-1}). In addition, NutriSphere-N®-coated urea was applied after the first and second cut (134.5 kg N ha^{-1}) and after the third cut (67.25 kg N ha^{-1}).

[4]For grass-clover, sodium selenate was applied May 5, 2017 and May 16, 2018. One day before Se application, fertilizer was applied including 0 (none), NPK (40 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1}, and 100 kg K_2O ha^{-1}), and NPKS (NPK plus 34 kg S ha^{-1}). In addition, urea (50 kg N ha^{-1}) was applied to the treated plots after each harvest.

[5]Selenium agronomic efficiency (%) = [(Se yield with selenate amendment – Se yield without selenate amendment)/amount of selenate amended] x 100.

[6]Ref. is designated as the unexposed control group (0 g Se ha^{-1}).

TABLE 4 Effect of springtime sodium-selenate foliar application rate (0, 45, and 90 g Se ha^{-1}) and nitrogen-phosphorus-potassium-sulfur fertilization (none, (N)PK, or (N)PKS) on annual forage N concentration, yield, and agronomic efficiency in forages across Oregon[1].

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
Year & Forage Type	**Average Annual N Concentration (forage DM %)**												
2017													
Alfalfa[2]	2.84	2.88	2.79	2.82	2.85	2.90	2.97	2.89	2.93	0.07	0.99	0.06	0.28
Grasses[2]	1.51	1.64	1.40	1.72	1.70	1.73	1.72	1.65	1.65	0.14	0.74	0.06	0.61
Orchard Grass[3]	1.41	1.45	1.39	1.83	1.80	1.88	1.76	1.73	1.72	0.05	0.94	<0.0001	0.38
Grass-Clover[4]	2.43	2.37	2.21	2.45	2.38	2.47	2.57	2.48	2.39	0.17	0.71	0.056	0.28
2018													
Alfalfa	2.61	2.65	2.68	2.74	2.68	2.62	2.74	2.68	2.72	0.12	0.90	0.53	0.75
Grasses	1.71	1.76	1.78	2.06	1.98	1.92	2.05	1.96	2.08	0.15	0.91	0.02	0.80
Orchard Grass	1.28	1.34	1.34	2.16[b]	2.26[ab]	2.39[a]	1.96	2.10	1.96	0.09	0.30	<0.0001	0.097
Grass-Clover	2.23	2.27	2.26	2.44	2.29	2.30	2.44	2.29	2.23	0.19	0.93	0.71	0.73
	Annual N Harvested (kg N ha^{-1})												
2017													
Alfalfa	299.0	307.6	310.5	354.8	343.8	369.7	356.4	362.5	343.5	13.87	0.76	0.0001	0.35
Grasses	31.67	65.15	26.42	68.68	61.59	62.42	83.91	76.27	60.20	22.37	0.58	0.02	0.41
Orchard Grass	48.87	62.84	48.37	192.4	182.5	182.2	208.6	201.7	202.9	13.44	0.88	<0.0001	0.40
Grass-Clover	81.43	77.03	87.54	133.5	128.0	129.0	135.0	134.7	130.3	14.65	0.96	<0.0001	0.88

(Continued)

TABLE 4 Continued

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
2018													
Alfalfa	318.1	309.3	305.6	385.6	367.2	397.0	407.2[a]	357.4[b]	376.2[ab]	21.86	0.16	0.0002	0.56
Grasses	24.84	76.63	39.38	102.9	80.98	85.90	101.6	91.51	83.62	33.25	0.88	0.01	0.35
Orchard Grass	51.68	65.90	55.35	209.1[b]	199.6[b]	251.6[a]	291.5	303.0	283.4	14.34	0.54	<0.0001	0.007
Grass-Clover	145.8	131.7	151.7	171.3	172.4	185.8	190.1	189.5	184.6	27.27	0.92	0.003	0.84
	Nitrogen Agronomic Efficiency (%)[5]												
2017													
Alfalfa	NA[6]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Grasses	Ref.[6]	Ref.	Ref.	25.70	23.27	25.01	36.28	33.46	23.46	11.42	0.79	0.24	0.55
Orchard Grass	Ref.	Ref.	Ref.	35.58	29.65	33.16	39.58	34.41	38.30	3.70	0.31	0.01	0.93
Grass-Clover	Ref.	Ref.	Ref.	37.19	36.40	29.39	38.25	41.21	30.56	11.54	0.65	0.63	0.93
2018													
Alfalfa	NA[6]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Grasses	Ref.[6]	Ref.	Ref.	54.19	41.41	32.30	53.31	48.72	30.72	17.34	0.40	0.81	0.83
Orchard Grass	Ref.	Ref.	Ref.	39.01[ab]	33.13[b]	48.63[a]	59.43	58.77	56.52	4.22	0.24	<0.0001	0.02
Grass-Clover	Ref.	Ref.	Ref.	18.17	29.10	24.38	31.60	41.29	23.47	18.71	0.71	0.42	0.80

[1]Data are shown as least-squared means (LSMs) of sodium selenate application rates within fertilization types with a pooled standard error of difference (SED) for each year. Forages were harvested 2 times (grasses both years and grass-clover in 2018) or 3 times (alfalfa and orchard grass both years and grass-clover in 2017) per year. LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with different lower-case superscripts are significantly different from each other (P≤0.05). LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with the same lower-case superscripts or no superscripts are not significantly different from each other (P≥0.05).

[2]For alfalfa and grasses at Union (eastern Oregon), sodium selenate (RETORTE Ulrich Scharrer GmbH, Röthenbach, Germany) was applied May 2, 2017 and 2018. Fertilizer was applied three days before selenate application in 2017 and on the day of selenate application in 2018 and included for alfalfa 0 (none), PK (34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and PKS (PK plus 12.0 kg S ha^{-1}) and for grasses 0 (none), NPK (72.0 kg N ha^{-1}, 34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and NPKS (NPK plus 12.0 kg S ha^{-1}). In addition, urea (72.0 kg N ha^{-1}) was applied after the first grass cut.

[3]For orchard grass at Terrebonne (central Oregon), sodium selenate was applied April 21, 2017 and May 4, 2018. Fertilizer was applied on the day of selenate application in 2017 and ten days before selenate application in 2018 and included 0 (none), NPK (134.5 kg N ha^{-1}, 33.6 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1}), and NPKS (NPK plus 33.6 kg S ha^{-1}). In addition, NutriSphere-N®-coated urea was applied after the first and second cut (134.5 kg N ha^{-1}) and after the third cut (67.25 kg N ha^{-1}).

[4]For grass-clover, sodium selenate was applied May 5, 2017 and May 16, 2018. One day before Se application, fertilizer was applied including 0 (none), NPK (40 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1}, and 100 kg K_2O ha^{-1}), and NPKS (NPK plus 34 kg S ha^{-1}). In addition, urea (50 kg N ha^{-1}) was applied to the treated plots after each harvest.

[5]Nitrogen agronomic efficiency (%) = [(N yield with NPK(S) application – N yield without NPK application)/amount of N applied] x 100.

[6]Ref. is designated as the unexposed control group: none. NA, not applicable.

Terrebonne had the highest Se agronomic efficiencies (25 to 37%), whereas grasses at Union and grass-clover at Roseburg had the lowest (5 to 17%). Similarly, forage Se concentrations and annual Se yields doubled from the 45 to the 90 g Se ha^{-1} amendment rate in high yielding forages (i.e., alfalfa at Union and orchard grass at Terrebonne) and less than doubled in low yielding forages (i.e. grass-clover at Roseburg in 2018 and not-fertilized and NPK-fertilized grasses at Union in 2017).

Selenium agronomic efficiency depended on the efficient conversion of N and Se into plant proteins. In support, Se agronomic efficiency (%) was strongly correlated to forage yield of Se (r = +0.85; $P < 0.0001$), forage N yield (r = +0.78; $P < 0.0001$), and N agronomic efficiency (r = +0.55; $P < 0.0001$; calculated by the formula [(N yield with NPK(S) fertilization – N yield without NPK fertilization)/amount of N applied] x 100), and forage nitrogen concentration (r = +0.49; $P < 0.0001$).

Foliar springtime selenate amendment at 45 and 90 g Se ha^{-1} did not affect annual forage N concentrations (all $P > 0.19$), N yields (all but one main effect $P > 0.28$), and N agronomic efficiencies (%) after N fertilization in Oregon in both years for all combinations of forages, sites, and S application rates (all main effects $P > 0.15$; Table 4).

Foliar springtime selenate amendment at 45 and 90 g Se ha^{-1} did not decrease forage S concentrations (all $P > 0.14$) nor S yields (all but one main effect $P > 0.30$; Table 5). The only exception was S yields of alfalfa at Union in 2018, when forage S yields were lower at 45 g Se ha^{-1} compared with the two other amendment rates combined ($P = 0.05$).

3.2 Response of Se-amended forages to NPK fertilizer

In Oregon, 2018 was a good forage production year, whereas 2017 was a lower forage production year. Almost all fertilizer, Se amendment, and forage site/species combinations produced more forage biomass in 2018 than in 2017 (Table 3) because of warmer temperatures in May 2018 than in May 2017.Without fertilization,

forage grass yields were very low. At Terrebonne and Union, selenate amendment at 45 g ha^{-1} increased forage yields of non-fertilized grass plots, but not among NPK-fertilized plots. There was a significant interaction between selenate amendment rate and NPK fertilization for orchard grass forage yield in 2018 (P = 0.02) and statistical tendencies for grasses forage yield in 2017 (P = 0.09) and 2018 (P = 0.07). Fertilization with NPK or PK (for alfalfa) increased annual forage yields (kg forage DM ha^{-1}), when compared with no fertilization (all main effects P < 0.03). The greatest forage yield increases were observed for orchard grass at Terrebonne in central Oregon (+ 4,000 to 6,000 kg forage ha^{-1}), whereas the other forage yield increases were similar in magnitude (+ 1,000 to 2,000 kg forage ha^{-1}).

Fertilization with NPK alone did not affect forage Se concentrations (Table 3; Figure 4). Co-application of selenate and (N)PK decreased forage Se concentrations at Terrebonne (both Se amendment rates P < 0.04), but not at the other two forage sites (all P > 0.19). Fertilization with NPK increased forage Se yields of orchard grass at Terrebonne in 2017 (P = 0.002) and 2018 (P = 0.02), grasses at Union in 2018 (P = 0.02), and grass-clover at Roseburg in 2017 (P = 0.03) and 2018 (P = 0.09). The largest increases in forage Se yields were observed for orchard grass at

TABLE 5 Effect of springtime sodium-selenate foliar application rate (0, 45, and 90 g Se ha^{-1}) and nitrogen-phosphorus-potassium-sulfur fertilization (none, (N)PK, or (N)PKS) on annual forage S concentration, yield, and agronomic efficiency in forages across Oregon[1].

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
Year & Forage Type	**Average Annual S Concentration (forage DM %)**												
2017													
Alfalfa[2]	0.210	0.210	0.230	0.250	0.277	0.260	0.263	0.250	0.260	0.013	0.73	<0.0001	0.02
Grasses[2]	0.190	0.183	0.183	0.203	0.193	0.213	0.193^{a}	0.167^{b}	0.180^{ab}	0.011	0.31	0.001	0.23
Orchard Grass[3]	0.170	0.170	0.173	0.153	0.150	0.157	0.193	0.187	0.180	0.011	0.90	0.0004	0.75
Grass-Clover[4]	0.307	0.310	0.283	0.333	0.303	0.333	0.330	0.337	0.320	0.018	0.74	0.005	0.069
2018													
Alfalfa	0.200	0.200	0.207	0.240	0.220	0.230	0.260	0.240	0.240	0.012	0.44	<0.0001	0.25
Grasses	0.170	0.167	0.160	0.183	0.167	0.180	0.180	0.193	0.190	0.010	0.93	0.006	0.23
Orchard Grass	0.167	0.173	0.170	0.133	0.133	0.143	0.157	0.160	0.157	0.007	0.63	<0.0001	0.53
Grass-Clover	0.297	0.313	0.330	0.293	0.307	0.307	0.307	0.317	0.330	0.019	0.30	0.30	0.92
	Annual S Harvested (kg S ha^{-1})												
2017													
Alfalfa	22.02	22.83	25.22	31.70	33.34	33.18	31.57	31.57	30.74	1.88	0.57	<0.0001	0.55
Grasses	3.92	6.40	3.43	8.11	7.05	7.62	9.19	7.58	6.50	1.81	0.62	0.004	0.26
Orchard Grass	5.85	7.46	6.06	15.98	15.30	15.17	22.78	21.88	21.23	1.42	0.79	<0.0001	0.35
Grass-Clover	10.21	10.12	11.24	17.72	16.27	17.28	17.27	18.38	17.56	1.40	0.91	<0.0001	0.55
2018													
Alfalfa	24.53	23.35	23.27	33.98^{ab}	30.58^{b}	34.87^{a}	38.25^{a}	31.91^{b}	33.47^{b}	1.68	0.09	<0.0001	0.02
Grasses	2.54	6.34	3.56	8.99	6.64	7.88	8.82	8.85	7.47	2.41	0.87	0.005	0.30
Orchard Grass	6.70	8.54	7.09	13.08	11.84	15.13	23.20	23.38	22.52	1.57	0.91	<0.0001	0.02
Grass-Clover	19.26	18.20	22.38	22.01	23.11	24.92	23.82	26.75	27.67	4.14	0.66	0.005	0.84
	Sulfur Agronomic Efficiency (%)[5]												
2017													
Alfalfa	NA[6]	NA	NA	Ref.[6]	Ref.	Ref.	-1.12	-14.75	-20.33	14.73	0.45	NA	NA
Grasses	NA	NA	NA	Ref.	Ref.	Ref.	8.98	4.42	-9.29	11.98	0.35	NA	NA
Orchard Grass	NA	NA	NA	Ref.	Ref.	Ref.	20.25	19.59	18.06	4.63	0.89	NA	NA

(Continued)

TABLE 5 Continued

	None			(N)PK			(N)PKS				P-values		
Se-Application Rate	0	45	90	0	45	90	0	45	90	SED	Se	Fertilizer	Se x Fert
Grass-Clover	NA	NA	NA	Ref.	Ref.	Ref.	-0.88	5.08	3.16	4.95	0.51	NA	NA
2018													
Alfalfa	NA[6]	NA	NA	Ref.[6]	Ref.	Ref.	35.55[a]	11.13[b]	-11.73[c]	9.11	0.006	NA	NA
Grasses	NA	NA	NA	Ref.	Ref.	Ref.	-1.46	18.42	-3.37	10.82	0.16	NA	NA
Orchard Grass	NA	NA	NA	Ref.	Ref.	Ref.	30.14	34.34	22.00	5.87	0.18	NA	NA
Grass-Clover	NA	NA	NA	Ref.	Ref.	Ref.	6.65	7.14	5.45	15.47	0.99	NA	NA

[1]Data are shown as least-squared means (LSMs) of sodium selenate application rates within fertilization types with a pooled standard error of difference (SED) for each year. Forages were harvested 2 times (grasses both years and grass-clover in 2018) or 3 times (alfalfa and orchard grass both years and grass-clover in 2017) per year. LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with different lower-case superscripts are significantly different from each other (P≤0.05). LSMs of forages within fertilizer type (none, (N)PK, or (N)PKS) with the same lower-case superscripts or no superscripts are not significantly different from each other (P≥0.05).

[2]For alfalfa and grasses at Union (eastern Oregon), sodium selenate (RETORTE Ulrich Scharrer GmbH, Röthenbach, Germany) was applied May 2, 2017 and 2018. Fertilizer was applied three days before selenate application in 2017 and on the day of selenate application in 2018 and included for alfalfa 0 (none), PK (34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and PKS (PK plus 12.0 kg S ha^{-1}) and for grasses 0 (none), NPK (72.0 kg N ha^{-1}, 34.0 kg P_2O_5 ha^{-1}, and 192.0 kg K_2O ha^{-1}), and NPKS (NPK plus 12.0 kg S ha^{-1}). In addition, urea (72.0 kg N ha^{-1}) was applied after the first grass cut.

[3]For orchard grass at Terrebonne (central Oregon), sodium selenate was applied April 21, 2017 and May 4, 2018. Fertilizer was applied on the day of selenate application in 2017 and ten days before selenate application in 2018 and included 0 (none), NPK (134.5 kg N ha^{-1}, 33.6 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1}), and NPKS (NPK plus 33.6 kg S ha^{-1}). In addition, NutriSphere-N®-coated urea was applied after the first and second cut (134.5 kg N ha^{-1}) and after the third cut (67.25 kg N ha^{-1}).

[4]For grass-clover, sodium selenate was applied May 5, 2017 and May 16, 2018. One day before Se application, fertilizer was applied including 0 (none), NPK (40 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1}, and 100 kg K_2O ha^{-1}), and NPKS (NPK plus 34 kg S ha^{-1}). In addition, urea (50 kg N ha^{-1}) was applied to the treated plots after each harvest.

[5]Sulfur agronomic efficiency (%) = [(S yield with (N)PKS application – S yield with (N)PK application)/amount of S applied] x 100.

[6]Ref. is designated as the unexposed control group: (N)PK. NA, not applicable.

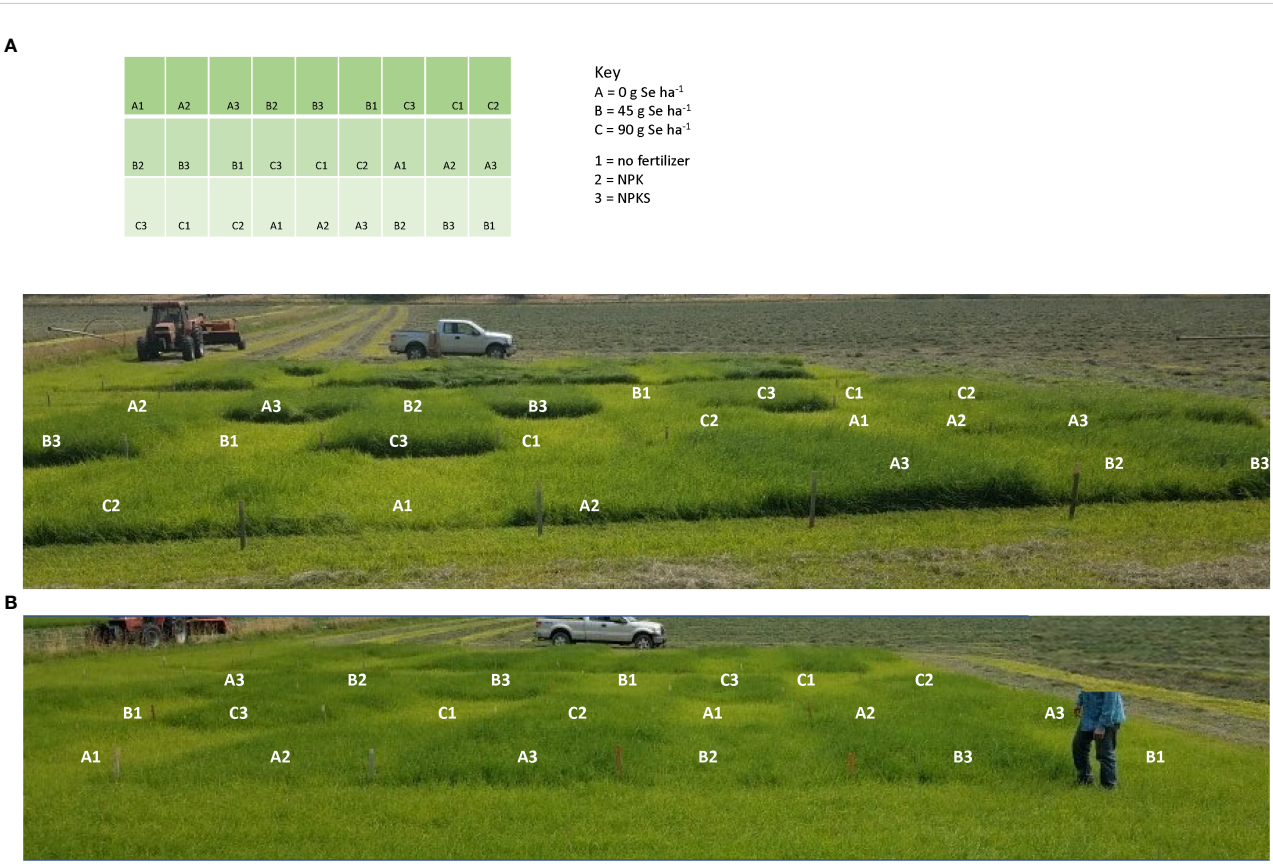

FIGURE 3
Forage plots at Terrebonne (central Oregon) in 2018 showing early growth of **(A)** second cutting, and **(B)** third cutting. Sodium selenate was applied to plots at rates of 0, 45, and 90 g Se ha^{-1} on May 4, 2018. Fertilizer was applied ten days prior to Se application, and consisted of no fertilizer, NPK (134.5 kg N ha^{-1}, 33.6 kg P_2O_5 ha^{-1}, and 112 kg K_2O ha^{-1}), or NPKS (NPK plus 33.6 kg S ha^{-1}). Forage growth shows the positive effects of S fertilization, whereas selenate amendment did not affect forage yields.

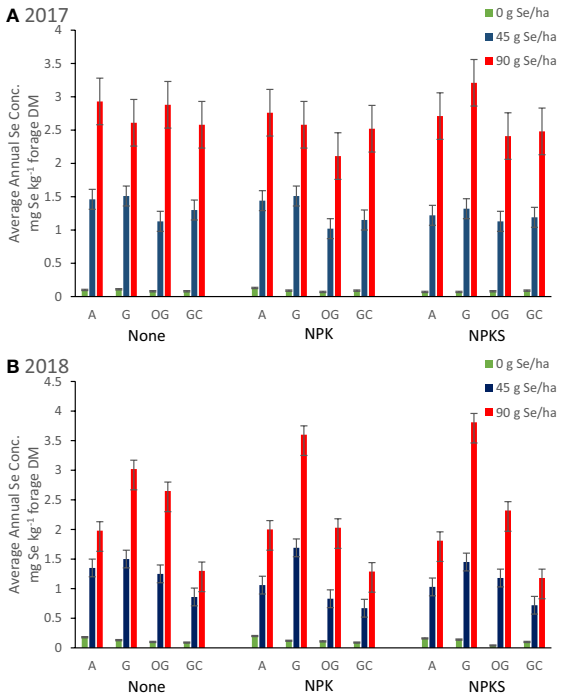

FIGURE 4
Average annual Se concentrations in forages (mg Se kg^{-1} forage DM) for plots in Union (eastern Oregon; alfalfa (A), and grass (G) mixture), Terrebonne (central Oregon; orchard grass, OG), and Roseburg (western Oregon; grass-clover mixture, GC) in **(A)** 2017 and **(B)** 2018. Sodium selenate was applied to plots at rates of 0, 45, or 90 g Se ha^{-1}. Fertilizer was applied as none, (N)PK, or (N)PKS at approximately the same time. Selenate amendment linearly increased forage Se concentrations (all $P < 0.004$), and were highly correlated to annual Se yield (g Se ha^{-1}; r = +0.70; $P < 0.0001$). Co-application of selenate and (N)PK decreased forage Se concentrations in S-deficient forage sites (Terrebonne; both Se amendment rates $P < 0.04$), but not at the other two forage sites (all $P > 0.19$). Sulfate-S application decreased forage Se concentrations, when plant availability of Se was low (0 g Se ha^{-1}; Terrebonne in 2018).

Terrebonne, as both NPK-fertilization and each additional 45 g ha^{-1} Se amendment increased Se yield by 5 g Se ha^{-1} to a total of 20 g Se ha^{-1} in NPK-fertilized and 90 g ha^{-1} Se amended plots (P interaction = 0.01 in 2017 and 0.02 in 2018). Co-application of selenate and NPK increased Se agronomic efficiency of grasses at Union in 2018 ($P = 0.05$), grass-clover at Roseburg in 2017 ($P = 0.03$), and orchard grass at Terrebonne in 2017 ($P = 0.02$) and in 2018 (only for 90 g ha^{-1} Se amendment). Fertilization with PK did not affect alfalfa Se concentrations and yields; co-application of selenate and PK did not affect Se agronomic efficiency (all $P > 0.17$).

NPK fertilization increased forage N concentrations (forage DM %) at Union for grasses in 2017 ($P = 0.03$) and in 2018 ($P = 0.02$), at Terrebonne for orchard grass in 2017 and 2018 (both $P < 0.0001$), and at Roseburg for grass-clover in 2017 ($P = 0.09$; only for 90 g ha^{-1} Se amendment; Table 4). No effect of PK fertilization on forage nitrogen concentrations was observed for alfalfa at Union ($P > 0.70$).

Fertilization with (N)PK increased annual forage nitrogen yields (kg N ha^{-1}), when compared with no fertilization (all main effects $P < 0.05$; Table 4; Figure 5). At Terrebonne, co-application of NPK and 90 g ha^{-1} Se amendment increased N yields more compared with the two lower Se amendment rates (P interaction < 0.04). Nitrogen agronomic efficiencies were consistently positive (18-59%; Table 4). Compared with Se agronomic efficiencies, N agronomic efficiencies were higher and, with exception of orchard grass at Terrebonne, more variable than Se agronomic efficiencies (Tables 3, 4). Both Se and N agronomic efficiencies depended on total forage yield (r = +0.45; P <0.0001). Orchard grass at Terrebonne in 2018 had the highest N agronomic efficiencies (33-49%), whereas grasses at Union in 2017 (23-26%) and grass-clover at Roseburg in 2018 (18-29%) had the lowest (Table 4).

The effect of (N)PK fertilization on forage S concentration depended on forage site and year (Table 5). At Terrebonne, NPK fertilization decreased forage S concentrations in both years, whereas forage S concentrations were increased at Union and at Roseburg in 2017. At all forage sites, NPK fertilization increased forage S yields (all main effects $P < 0.04$; Table 5). Co-application of NPK and 90 g Se ha^{-1} resulted in the highest S yields in 2018 at Terrebonne (P interaction = 0.005) and for alfalfa and grasses at Union (both P interactions = 0.05).

3.3 Response of Se-amended forages to NPK plus S fertilizer

With the exception of orchard grass at Terrebonne, (N)PKS fertilization did not increase forage yields compared with (N)PK fertilization (all main effects $P > 0.16$; Table 3; Figure 3). At Terrebonne, S fertilization increased forage yield (kg forage DM ha^{-1}) in 2017 by 1,000 kg forage ha^{-1} ($P = 0.0004$) and in 2018 by 5,000 kg forage ha^{-1} ($P < 0.0001$); these forage yields were similar to PK- and PKS-fertilized alfalfa plots at Union. At Terrebonne, selenate amendment at 90 g ha^{-1} increased forage yields of NPK-fertilized grass plots in 2018, but not among NPKS-fertilized plots.

There was a significant interaction between selenate amendment at 90 g ha^{-1} and S fertilization for orchard grass forage yield at Terrebonne in 2018 (P = 0.01).

(N)PKS fertilization without Se amendment decreased forage Se concentrations in high-producing forages (Table 3; Figure 4). Co-application of NPKS and selenate at 90 g ha^{-1} increased Se concentrations of grass-based forages in Union and in Terrebonne. At Terrebonne, co-application of NPKS and selenate increased harvested Se yields in 2017 (P = 0.03) and even more so in 2018 (P = 0.0007; Figure 6). In addition, co-application of NPKS and selenate increased Se agronomic efficiency (%) at Terrebonne in 2017 by 5 to 8% (P = 0.09) and in 2018 by 14 to 22% (P = 0.0005).

The effect of S fertilization on forage N concentrations differed by forage species and year (Table 4). Sulfur fertilization increased annual forage N concentrations for alfalfa at Union in 2017 (P = 0.05) and decreased it for orchard grass at Terrebonne in 2017 (P = 0.005) and 2018 (P < 0.0001). Despite decreasing forage DM N concentration, S fertilization increased forage N yield for orchard grass at Terrebonne in 2017 (P = 0.004) and 2018 (P < 0.0001) (Figure 5).

Orchard grass was the only forage species in which N agronomic efficiency was affected by S fertilization (all other P > 0.23). Sulfur fertilization of orchard grass at Terrebonne increased N agronomic efficiency (2017: P= 0.01; 2018: P < 0.0001), with the greatest responses observed for co-application of NPKS and 90 g Se ha^{-1}).

The effect of S fertilization on forage S concentrations (forage DM %) and yields (kg S ha^{-1}) differed by year and forage (Table 5). At Terrebonne, S fertilization increased forage S concentrations (2017: P = 0.0004; 2018: P < 0.0001) and yields (both P < 0.0001). At Union, S fertilization decreased forage S concentrations in 2017 (for grasses; P = 0.0005), but increased forage S concentrations in 2018 (for alfalfa: P = 0.005; for grasses: P = 0.07). Sulfur fertilization without Se amendment increased S yield of alfalfa at Union in 2018 (P interaction = 0.03; Figure 6). No changes were observed in Roseburg (all P > 0.13).

Sulfur agronomic efficiency was positively correlated with forage yield (r = +0.41; P = 0.0003) and negatively correlated with forage concentrations of N (r = -0.32; P = 0.006), S (r = -0.31; P = 0.007), and Se (r = -0.29; P = 0.01). Terrebonne was the only forage site consistently responsive to S fertilization (range of S agronomic efficiencies: 18-34%) and having a low variability (SED < 6%). In 2018, alfalfa was responsive to S fertilization without Se amendment (36%), but not with Se amendment.

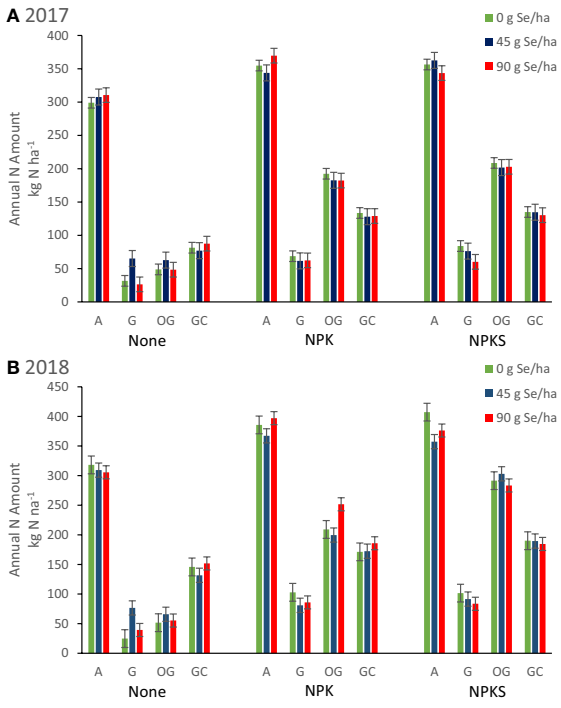

FIGURE 5
Annual nitrogen harvested from forages (kg N ha^{-1}) for plots in Union (eastern Oregon; alfalfa (A), and grass (G) mixture), Terrebonne (central Oregon; orchard grass, OG), and Roseburg (western Oregon; grass-clover mixture, GC) in **(A)** 2017 and **(B)** 2018. Sodium selenate was applied to plots at rates of 0, 45, or 90 g Se ha^{-1}. Fertilizer was applied as none, (N)PK, or (N)PKS at approximately the same time. Sulfur fertilization increased forage N yield for orchard grass at Terrebonne in 2017 (P = 0.004) and 2018 (P < 0.0001).

4 Discussion

4.1 Forage requirements

Forages require light, water, heat, and nutrients for growth. We examined two forage grasses (i.e., orchard grass, and a grass mixture containing primarily tall fescue and orchard grass), one legume (i.e., alfalfa), and a grass-legume (50%:50%) mixture containing tall fescue, ryegrass, orchard grass and clovers). Forages were grown in varying climatic sites: Roseburg in southwestern Oregon, Terrebonne in central Oregon, and Union in eastern Oregon. In eastern, and even more so in central Oregon, the growing season is short and rain fall is limited and occurs mainly outside the growing season, requiring snow melt from the mountains to provide sufficient water in spring and irrigation during summer. Roseburg has a longer growing season, more precipitation, but requires irrigation in the summer. Cold, wet spring conditions can delay forage growth, which happened in May 2017.

Forages rely on plant availability of macro- and micro-nutrients in adequate amounts. The three most important macro-nutrients are N, P, and K (Tripathi et al., 2014). Nitrogen is a structural part of chlorophyll and plant proteins, most of which are in the leaves. Besides providing structure, plant proteins regulate water and nutrient assimilation, and thus are essential for plant growth (Masclaux-Daubresse et al., 2010). Grass-based forages are more susceptible to N deficiency than legume-based forages such as alfalfa, because forage legumes can convert N gas to ammonium-N compounds with the help of bacteria in the root region (Russelle et al., 1994; Moore et al., 2019). As expected, grass-based, unfertilized forages showed N deficiencies in both forage production years; the latter was indicated by low forage production, poor grass morphology (pale green color of leaves), and low forage grass DM N concentrations (<2-2.5% plant DM) (Moore et al., 2019). We conclude that grass-based forages across

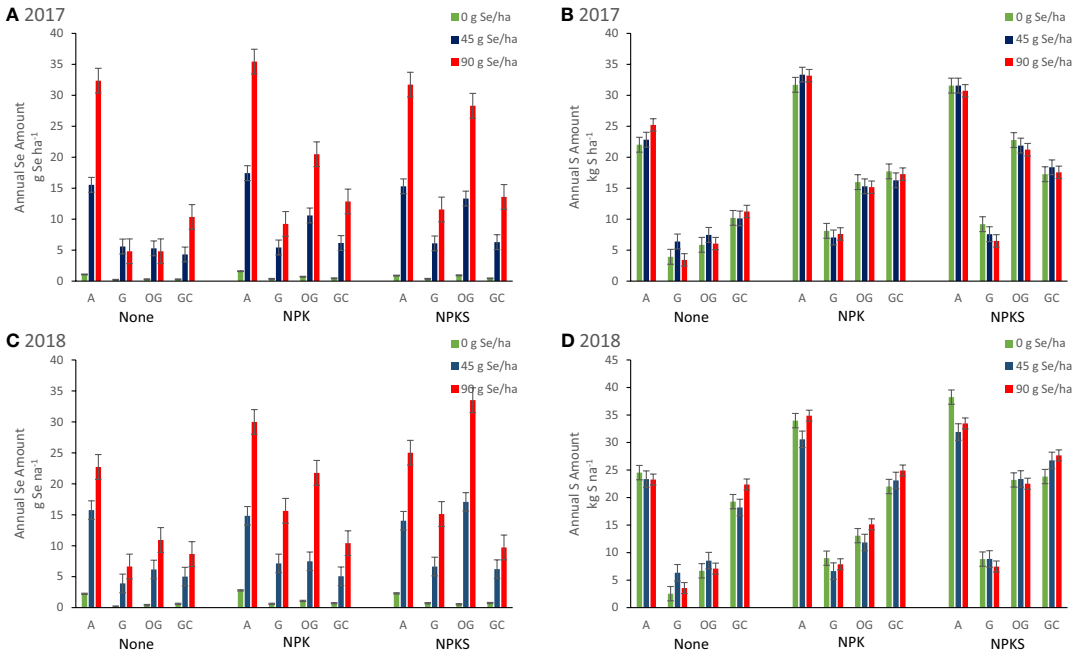

FIGURE 6
Annual Se (g Se ha^{-1}) and S (kg S ha^{-1}) harvested from forages in Union (eastern Oregon; alfalfa (A), and grass (G) mixture), Terrebonne (central Oregon; orchard grass, OG), and Roseburg (western Oregon; grass-clover mixture, GC) in **(A, B)** 2017, and **(C, D)** 2018, respectively. Sodium selenate was applied to plots at rates of 0, 45, or 90 g Se ha^{-1}. Fertilizer was applied as none, (N)PK, or (N)PKS at approximately the same time. Sulfur fertilization without Se amendment increased S yield of alfalfa at Union (*P* interaction = 0.03). At Terrebonne, co-application of NPKS and selenate increased harvested Se yields in 2017 (*P* = 0.03) and even more so in 2018 (*P* = 0.0007). At Roseburg, higher annual S yield from GC in 2018 in the absence of S fertilization (S was present in the irrigation water) was associated with lower annual Se yield after selenate amendment.

Oregon require N fertilization to prevent forage N deficiency. Furthermore, N fertilization is required to provide sufficient forage biomass for livestock.

Sulfur concentrations of unfertilized forages indicated that soil S availability was high in Roseburg, adequate in Union, and low in Terrebonne. Consequently, orchard grass at Terrebonne, but not the other two sites, required S fertilization. Roseburg's soils are traditionally low in S because of their low sulfate-holding capacity; consequently, we were surprised about the high forage S concentrations at Roseburg. The S content of soil in Roseburg (western Oregon) is not traditionally measured because high rainfall makes it highly variable and single point measurements are not commonly used to advise the need for S fertilization. We did submit a soil sample from the Roseburg site to the same laboratory where S concentrations were determined in forage samples (Soil Health Laboratory at Oregon State University, Corvallis, OR). These results showed sulfate-S was 3.05 mg/kg. Oregon State University recommended soil sulfate-S concentrations are only given for alfalfa (15 mg/kg) and not grass or grass-clover forage crops. Based on a recommendation of 15 mg sulfate-S for grass and grass-legume forage crops, then Roseburg soils are S deficient. We also investigated the irrigation water for S content in Roseburg by reviewing data from the Oregon Department of Environmental Quality (AWQMS database) for average S concentrations upriver that was closest to the forage plots in Roseburg during summer irrigation season. The historical (1997 – 2022) average S concentrations was 6.68 mg sulfate/L, or 0.14 mEq/L. Using calculations for application of sulfate-S by irrigation water (Hopkins et al., 2007), this is equivalent to 14.5 kg ha^{-1} of S applied through the irrigation water. In Roseburg, the NPKS fertilization rate was 34 kg S ha^{-1}. Thus, irrigation water resulted in approximately 50% of the recommended sulfate-S application. This explains why the high grass-clover S content in Roseburg indicated adequate S availability. At Union, unfertilized alfalfa had higher S concentrations than unfertilized grasses, which is consistent with the fact that alfalfa forages generally have higher macronutrient concentrations than grass forages (Saito, 2004).

Selenium concentrations of non Se-amended soil and forages were low across Oregon, which is consistent with findings in our previous studies (Oldfield, 2001; Hall et al., 2009; Hall et al., 2011a; Hall et al., 2013a; Brummer et al., 2014). We previously reported that Se concentrations of non Se-amended forages, similar to those observed in this report, results in low whole-blood Se concentrations and, consequently, poor health and growth of livestock consuming those forages (Hall et al., 2011b; Stewart et al., 2012; Hall et al., 2013a; Hall et al., 2013b; Hugejiletu et al., 2013; Hall et al., 2014a; Hall et al., 2014b; Hall et al., 2017; Hall et al., 2020). Forages assimilate soil Se species with different efficiencies (White, 2016). Selenate is highly mobile in the soil and is efficiently absorbed by the roots *via* high-affinity sulfate transporters (Pickering et al., 2000; White, 2018). Selenate is converted by soil microbes to SeMet and SeCys, which can be efficiently absorbed by the roots *via* amino acid membrane transporters (Pickering et al., 2000; White, 2018). Selenite is tightly bound to soil matter and

cannot be easily absorbed by the roots (Pickering et al., 2000; White, 2018). Elemental Se or methylated Se cannot be absorbed by the roots (Pickering et al., 2000; White, 2018). Consequently, only a portion of soil Se species are available for Se assimilation by the plant. As a result, forage Se concentrations were lower than soil Se concentrations. We conclude that forage Se concentrations are a better indicator of plant available Se than soil Se concentrations.

4.2 Effects of selenate amendment on forage nutrient contents and Se amendment utilization

Springtime application of foliar sodium selenate amendment did not affect annual yields of forages (i.e., alfalfa, orchardgrass, or other grass mixtures) grown across Oregon. This agrees with previous studies performed by others when rates of up to 100 g of Se ha^{-1} were applied (reviewed by (Ramkissoon et al., 2019) showing that Se is not an essential element for plants (White, 2016). Applying Se as an amendment increases Se concentrations in the edible portions of crop plants. Selenium agronomic biofortification of plants has proven to be both effective and safe for alleviating Se deficiencies in human and livestock populations in many countries, e.g., Finland, New Zealand, Australia, United Kingdom, and others (Whelan et al., 1994; Makela et al., 1995; Broadley et al., 2006; Ramkissoon et al., 2019).

Despite being non-essential for plants, Se uptake may benefit plant growth and survival, e.g., by conferring tolerance to environmental factors associated with oxidative stress, and by providing resistance to pathogens and herbivory (Quinn et al., 2007; Pilon-Smits et al., 2009; White and Brown, 2010; El Mehdawi and Pilon-Smits, 2012; Feng et al., 2013; White, 2016). In our study, selenate amendment at 45 g ha^{-1} increased forage biomass of non-fertilized grasses at Union and Terrebonne. We conclude that Se-amendments may benefit growth of grass pastures that are low in plant available macro-nutrients.

We showed in our previous report (Wang et al., 2021) that forage Se concentrations increased linearly from 2.06 to 4.15 mg kg^{-1} forage dry matter (DM) with doubling of foliar sodium selenate application rates from 45 to 90 g Se ha^{-1}. We concluded that selenate amendment rates can be used to predict forage Se concentrations. In turn, feeding selenate amended forages to livestock can prevent Se-deficiency.

Of the absorbed selenate, up to 82% was converted to SeMet in the forage, indicating efficient conversion of selenate to SeMet in plants (Wang et al., 2021). The conversion of selenate to SeMet depended on forage growth and time span between Se amendment application and forage harvest. Almost all the incorporated Se was removed during the growing season with forage harvesting (87% and 9% in the first and second cuts, respectively), indicating a limited selenate holding capacity of the soils. Grass-based forages had greater increases in Se concentrations in the first two cuts after selenate amendment compared with legume-based forages.

In this report, we calculated Se agronomic efficiencies after selenate amendment (indicative of Se utilization by the plant). The variability of Se agronomic efficiencies was low, indicating that the foliar spraying application was uniform. We conclude that application of foliar selenate amendment is consistent in improving forage Se concentrations.

We also showed that Se uptake increased with forage growth. The four forages (i.e., alfalfa, grass mixtures, orchard grass, and grass-clover) are non Se-accumulating forages (cannot tolerate tissue Se concentration >10 to 100 μg Se g^{-1} plant DM) (White, 2016). Non Se-accumulating forages have three Se pools in the plant: vacuole-stored selenate, proteins containing selenoamino acids (SeAA), and non-proteinogenic organo-selenium compounds (e.g., methylated selenocysteine). Plants usually absorb inorganic selenate either through the roots or through the leaf stomata if foliar applied. The inorganic selenate pool serves as a reserve pool for SeAA synthesis, which is promoted by plant protein synthesis (Pickering et al., 2000; White, 2018). Our previous study indicated that the inorganic selenate pool is limited to approximately 5 mg Se kg^{-1} forage DM, and cannot be increased, for example by a 10× higher selenate amendment rate than used in the current study (Hall et al., 2023). In this report, Se utilization by the plant was not altered by selenate amendment rate, indicating an efficient absorption of selenate by the plant. Furthermore, selenate was efficiently converted in the plant to SeMet (Wang et al., 2021). This conversion requires N-containing enzymes. We conclude that Se incorporation into forage plants depends on efficient conversion of N and Se into plant proteins, which, in turn, depends on weather conditions, macronutrient availability, and forage species.

Selenate amendment did not impact concentrations and yields of N and S in non-fertilized forages. This was not surprising given that much lower amounts of selenate were applied compared with plant available amounts of S and N in the soil. Moreover, selenate was foliar amended rather than soil amended, facilitating leaf absorption of selenate.

4.3 Effects of NPK or PK fertilization on forage nutrient contents and fertilizer utilization

Fertilization with NPK or PK (for alfalfa) increased biomass yields of forages grown across Oregon in both years. Forages require N for synthesis of nucleotides, ATP, proteins, and chlorophyll (Masclaux-Daubresse et al., 2010). The largest biomass increases were observed for orchard grass at Terrebonne, the forage site with the lowest soil nitrate concentrations in 2017. Forage grasses utilize more N during the early vegetative stages for tillering than legumes do for early shoot growth (Masclaux-Daubresse et al., 2010; Moore et al., 2019). Orchard grass received the most N fertilizer (403 kg N ha^{-1} *vs.* <145 kg N ha^{-1} at the other sites). We conclude that NPK or PK (for alfalfa) fertilization increases forage productivity in Oregon.

Nitrogen fertilization rates were adapted to meet local forage and soil nitrogen requirements. Nitrogen is primarily applied in the form of ammonium and nitrate compounds. We used granular ammonium phosphate in spring and urea or slow-release urea after each forage cut for N fertilizer. Ammonium ion and ammonia, the urease hydrolysis products of urea, are primarily absorbed by the roots through ammonium- and ammonia-specific transmembrane

proteins (Masclaux-Daubresse et al., 2010). Ammonium can only be stored in limited amounts in vacuoles before it is toxic to plants. Consequently, ammonium ion is rapidly transported to the leaves, where it is incorporated into DNA, ATP, and amino acids. N fertilizer rates met soil N requirements, shown best by soil nitrate concentrations in 2018. Based on forage N concentrations and plant morphology, N fertilization rates also met plant nitrogen requirements. Nitrogen fertilizer increased the (inadequate) forage N concentrations of non-fertilized forage grasses at Union (144 kg N ha^{-1} total application rate) and at Terrebonne (403 kg N ha^{-1} total application rate), but not the (adequate) forage N concentrations of non-fertilized forage legumes for grass-clover at Roseburg (140 kg N ha^{-1} total application rate) and alfalfa at Union (0 kg N ha^{-1} total application rate). Nonetheless, N fertilizer amounts were not enough to raise N concentrations of fertilized grasses to levels that would meet crude protein needs (15 to 18%) for livestock consuming the forages at Union and at Terrebonne. Our data support previous studies (Hart et al., 2000; Pirelli et al., 2004; Moore et al., 2019) showing that regular testing of forage N concentrations can aid in determining appropriate N fertilization rates for increasing forage production and N concentrations.

In this study, we calculated N agronomic efficiency after fertilization as an indication of N uptake and utilization. We showed that N fertilization consistently increased forage N uptake, and resulted in increased harvested forage N amounts after fertilizer treatment. Based on the positive correlation between forage yield and N agronomic efficiency, fresh forage growth promotes N fertilizer uptake, and more ammonium fertilizer is incorporated into forage proteins. Fresh forage growth depends on the photosynthetic capacity of forages, which, in turn, depends on light, water, sufficient leaf cover, and warm soil temperatures. We conclude that weather conditions have to be considered when determining N fertilization rates for forage production.

Fertilization with NPK or PK increased uptake and utilization of S in forages and increased harvested amounts of forage S. Sulfur is primarily absorbed by sulfate-specific transmembrane proteins in the root in the form of sulfate and then transported to the leaves (Saito, 2004). In the leaves, sulfate is converted into S-containing amino acids (i.e., Cys and Met) for protein synthesis. The correlation results indicate that forage growth promotes sulfate uptake and synthesis of Cys and Met to meet the plant S requirements. Fertilization with NPK increased sulfate uptake in grasses even more so in combination with 90 g ha^{-1} Se amendment, suggesting that the higher application rate of foliar selenate amendment may promote transmembrane protein synthesis in the roots for sulfate absorption when there is inadequate S available to the plant. Fertilization with NPK or PK fertilizers increased forage S concentrations in S-sufficient plant environments (i.e., Roseburg and Union) and lowered forage S concentrations in S-deficient plant environments (i.e., Terrebonne), indicating that NPK or PK fertilization only increased forage S concentrations when sufficient sulfate was available to roots.

Fertilization with NPK increased harvested amounts of forage Se by increasing selenate uptake and metabolism. Foliar selenate amendment is taken up by the leaves and converted into Se-containing amino acids (i.e., SeCys and SeMet) for protein synthesis (White, 2016). Correlation results indicate that forage growth promotes foliar selenate uptake and utilization by the plants. Co-application of NPK and 90 g ha^{-1} Se amendment resulted in the largest increases in forage Se yields. At Terrebonne, NPK-fertilization and each additional 45 g ha^{-1} Se amendment increased Se yield by 5 g Se ha^{-1} plant DM to a total of 20 g Se/kg plant DM in NPK-fertilized and 90 g ha^{-1} Se amended plots. We hypothesize, and our data supports, that in the absence of sufficient plant available S, Se can act as a S substitute making selenocysteine instead of cysteine and selenomethionine instead of methionine for protein synthesis.

Forage Se concentrations depended on both plant response to (N)PK fertilization and plant available Se concentrations. Fertilization with NPK did not affect forage Se concentrations in the absence of Se amendment, indicating that NPK fertilization promoted Se uptake by the roots. Fertilization with NPK or PK fertilizers did not affect forage Se concentrations in selenate-amended S-sufficient forage sites (i.e., Roseburg and Union), suggesting that NPK or PK fertilizers promoted foliar selenate amendment uptake. In contrast, NPK-fertilization plus selenate amendment lowered forage Se concentrations in S-deficient forage sites (i.e., Terrebonne). It is possible that higher selenate amendment rates are needed to increase forage Se concentrations in S deficient sites.

4.4 Effects of NPKS or PKS fertilization on forage nutrient utilization

Co-application of S with NPK or PK (for alfalfa) only increased biomass yields of orchard grass at Terrebonne in central Oregon, which had the lowest plant S concentrations at baseline. Sulfur is considered the fourth most important macro-nutrient (behind NPK) with many essential functions (Tripathi et al., 2014). For example, S-containing peptides and proteins are important for chlorophyll synthesis and function; for legume root nodule formation needed for N gas assimilation; for synthesis of amino acids, enzymes, and vitamins; for plant detoxification processes; for redox chemistry; and for disease resistance (Saito, 2004; Zenda et al., 2021).

Sulfur is becoming more important as a limiting nutrient in forage production, as S dioxide concentrations in the air have decreased over the last several decades by over 90%. Terrebonne and Roseburg are more susceptible to plant S deficiency because of their coarse soil texture (>50% sand content) and low organic matter content (<2%), which makes soils susceptible to sulfate leaching. Thus, we applied more S in Terrebonne and Roseburg (34 kg S ha^{-1}) than in Union (12 kg S ha^{-1}). The resulting high grass-clover S content in Roseburg indicated adequate S availability, from irrigation water as well as S fertilizer.

The only biomass yield increases observed for NPKS-fertilized orchard grass were at Terrebonne, the forage site that received the most N fertilizer (403 kg N ha^{-1} *vs.* <145 kg N ha^{-1} at the other sites) and the site that had the lowest soil nitrate concentrations in 2017. Forage requirement for S and N are closely associated, as both are required for protein and chlorophyll formation and function, as

well as for absorption and assimilation of nutrients (Saito, 2004; Tripathi et al., 2014; Zenda et al., 2021). We used ammonium sulfate and gypsum (hydrated calcium sulfate) for S application. Sulfur application rates were based on forage production goals; soil and forage analyses for S; soil texture and organic matter content; NPK fertilization rates; and weather conditions. We conclude that NPKS fertilization is necessary for maximizing forage production in Se depleted soils with limited plant sulfate availability, but with large forage growth potential.

Co-application of sulfate and NPK only increased forage sulfate concentrations when weather conditions were optimal for forage growth, and/or when plant sulfate availability was limited by the soil, by high forage growth rate, or by both. At Roseburg, despite limited soil sulfate availability because of sandy soil texture, low organic matter content and low CEC capacity, sulfate fertilization did not affect the already high forage S concentrations. Sulfur fertilizer uptake was below 8% with no changes in S yield, indicating no need for sulfate fertilization beyond what was applied *via* irrigation water. At Terrebonne, sulfate fertilization increased orchard grass S concentrations and yield. Sulfur fertilizer uptake was at least 18% and was higher in 2018 than in 2017. However, S concentrations of NPKS-fertilized orchard grass remained under 2%, which indicated that higher sulfate application rates than were applied are needed in high forage production years. At Union, where soil had higher organic matter and less sand, sulfate application increased alfalfa and grasses sulfate concentrations in 2018 but not in 2017, indicating that sulfate application may be needed in high forage production years, but not in low forage production years such as in 2017.

The co-dependency of S and N for forage uptake was exemplified by the effect of S application on forage N concentrations and N uptake as well as the high correlation between the forage S and N concentrations. At Terrebonne, sulfate fertilization increased N fertilizer uptake by orchard grass. The decrease in orchard grass N concentrations indicated that higher NPK fertilizer rates were needed to optimize forage growth and quality in high forage production years such as in 2018. We conclude that S fertilization increases N requirements in low plant available S soils. At Roseburg, sulfate application did not affect forage N concentrations and N uptake, indicating that sulfate application was not needed in either forage production year, as sufficient sulfate was available *via* irrigation water. At Union, S application had minimal effects on alfalfa N concentrations and N uptake, as sufficient sulfate was available in the soil.

Of great concern is the competition between sulfate and selenate for root uptake in forages (Moore et al., 2019). Selenate is highly mobile in soil and highly available to plants, being taken up *via* the same high-affinity sulfate-S membrane transporters (Li et al., 2008; Luo et al., 2019). In our study, S application decreased forage Se concentrations, when plant availability of Se was low. Similarly, Stroud et al. (Stroud et al., 2010) showed in wheat that S fertilization decreased Se-uptake in the absence of Se-application. One potential reason for this is that S supplementation alone may decrease the expression of genes encoding plant S transporters (Stroud et al., 2010; Boldrin et al., 2016; White, 2018). Another potential reason is that sulfate fertilizer out-competes soil selenate for root uptake. We conclude that sulfate fertilization decreases forage Se concentration of non-selenate amended fields.

In our study, the effect of co-application of foliar selenate with NPKS on forage selenate uptake depended on forage available S and/or forage species. At Terrebonne, S fertilization increased selenate amendment uptake in both years. Similarly, other researchers have shown that co-application of foliar Se with NPKS fertilizers doubled the Se concentration in wheat grains compared with application of foliar Se alone (Ramkissoon et al., 2019). One potential reason is that increased forage growth in response to S fertilization may promote foliar selenate uptake and/or utilization. Sulfur fertilization did not affect selenate amendment uptake or forage Se concentrations at the other two forage sites where S fertilization was not required. Of concern were the lower forage Se concentrations, lower annual Se yields, and lower Se agronomic efficiencies after selenate amendment at Roseburg in 2018, which was associated with higher forage S concentrations and S yields even without S fertilization at that site. We conclude that excess sulfate application *via* irrigation water may be detrimental to forage Se concentrations in selenate amended plots.

5 Conclusion

Application of foliar selenate amendment increases forage Se concentration based on amendment rates, irrespective of forage fertilization practices. Plant uptake of N and S from fertilizers did not interfere with plant uptake of selenate amendment. In fact, foliar selenate amendment synergizes with NPK(S) fertilization in promoting forage biomass production and plant uptake of N and S from fertilizer to satisfy nutrient requirements. However, S fertilizers can decrease forage Se concentrations, when plant available Se is already low and no selenate is amended. We have shown that multiple factors affect forage Se concentrations and Se yields including selenate amendment rate, the amount of forage biomass produced, forage species of interest, soil characteristics, and changing weather conditions from year to year. Because selenate amendment and S application cost extra, and S has the capacity to acidify soils, their concentrations in soil and plants should be routinely measured before application. Combining springtime sodium selenate foliar application with NPKS/PKS fertilizers at amounts adapted to meet local forage and soil requirements is an effective strategy to maintain optimal forage growth and quality on low Se soils in Oregon.

Data availability statement

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

Author contributions

JH: Conceptualization, Project Administration, Investigation, Formal analysis, Writing - Original Draft. GB: Data Curation, Formal

analysis, Writing - Original Draft, Visualization. SF: Investigation, Writing - Review and Editing. GP: Conceptualization, Funding acquisition, Project Administration, Writing - Review and Editing. MB: Investigation, Funding acquisition, Supervision, Writing - Review and Editing. GW: Investigation, Writing - Review and Editing. TD: Investigation, Resources, Writing - Review and Editing. GB: Investigation, Resources, Validation, Writing - Review and Editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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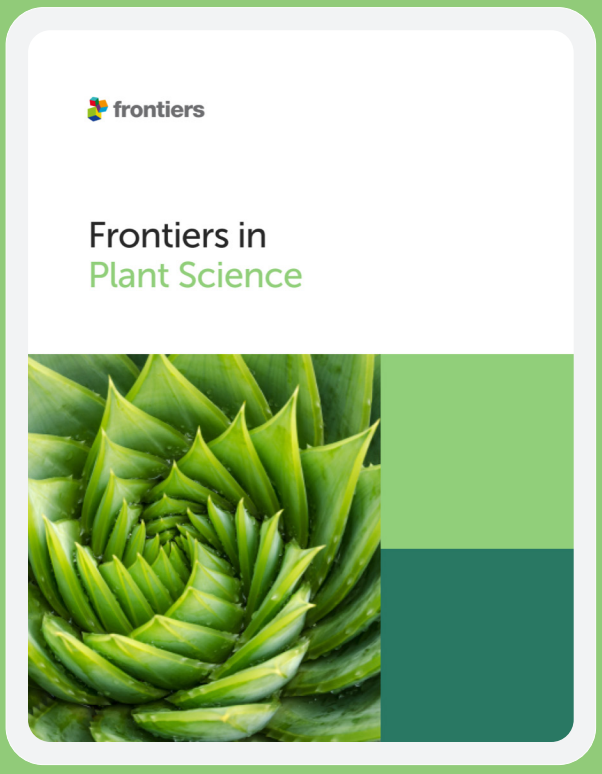

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