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Direct and indirect selenium speciation in biofortified wheat: A tale of two techniques

Maria Angels Subirana[1] | Roberto Boada[1] | Tingting Xiao[1] | Mercè Llugany[2] | Manuel Valiente[1]

[1]GTS-UAB Research Group, Department of Chemistry, Faculty of Science, Universitat Autònoma de Barcelona, Bellaterra, Spain

[2]Plant Physiology Group (BABVE), Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Spain

Correspondence
Mercè Llugany, Plant Physiology Group (BABVE), Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.
Email: merce.llugany@uab.cat

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Abstract

Wheat can be biofortified with different inorganic selenium (Se) forms, selenite or selenate. The choice of Se source influences the physiological response of the plant and the Se metabolites produced. We looked at selenium uptake, distribution and metabolization in wheat exposed to selenite, selenate and a 1:1 molar mixture of both to determine the impact of each treatment on the Se speciation in roots, shoots, and grains. To achieve a comprehensive quantification of the Se species, the complementarity of high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry and X-ray absorption spectroscopy was exploited. This approach allowed the identification of the six main selenium species: selenomethionine, selenocysteine, selenocystine, selenite, selenate, and elemental selenium. The three treatments resulted in similar total selenium concentration in grains, 90–150 mg Se kg^{-1}, but produced different effects in the plant. Selenite enhanced root accumulation (66% of selenium) and induced the maximum toxicity, whereas selenate favored shoot translocation (46%). With the 1:1 mixture, selenium was distributed along the plant generating lower toxicity. Although all conditions resulted in >92% of organic selenium in the grain, selenate produced mainly C-Se-C forms, such as selenomethionine, while selenite (alone or in the mixture) enhanced the production of C-Se-Se-C forms, such as selenocystine, modifying the selenoamino acid composition. These results provide a better understanding of the metabolization of selenium species which is key to minimize plant toxicity and any concomitant effect that may arise due to Se-biofortification.

1 | INTRODUCTION

Selenium (Se) is an essential micronutrient for humans since it is found as selenocysteine in 25 human selenoproteins (D'Amato et al., 2020). This amino acid is located in the active sites of enzymes and performs catalytic redox reactions, supporting various physiological functions (Rayman, 2000). Although Se has a predominant role in antioxidant activity, it also contributes to mitigate several pathophysiological conditions (e.g., heart disease, type-2 diabetes, neuromuscular disorders, depression) (Rayman, 2000); it is involved in the functioning of the immune system and the production of the active thyroid hormone (Rayman, 2000), it is effective in the chemoprevention of specific cancers (Weekley & Harris, 2013), and it may inhibit viral expression and virulence of HIV, influenza and COVID-19 (Gong et al., 2020; Rayman, 2012; Zhang et al., 2020). Hence, an appropriate Se dietary intake can highly benefit human health.

The Se content of food determines the Se status of the population, but the range of adequate consumption is narrow, and both excessive and deficient ingestion may lead to Se-related diseases and medical conditions. Regular consumption of food containing between 0.1 and 1 μg Se g^{-1} is considered adequate to reach the recommended 55 μg Se day^{-1} (Dumont et al., 2006). Unfortunately, cases of moderate deficiency are rather common, and it has been estimated that around one billion people may be Se deficient (Hawkesford & Zhao, 2007), with insufficient Se levels in their blood to optimize the glutathione peroxidase (GPx) enzymatic activity (Rayman, 2000). This can be attributed to low Se concentration in soils, which results in a Se shortage through the food chain. Crop enrichment with Se-containing fertilizers has been proposed as the best solution for Se-biofortification (Hawkesford & Zhao, 2007). Indeed, this strategy has already been successfully applied in Finland, where the Se level in blood serum of the population noticeably improved (Alfthan et al., 2015), as well as in the USA, the UK, Australia and China (Mora et al., 2015).

Although Se is not essential for land plants since it does not fulfill a specific role in their metabolism (Hawkesford & Zhao, 2007), plants can tolerate and even thrive on limited amounts of Se. Above a certain threshold, which depends on the plant accumulation capacity, toxic effects may appear, which can depress plant growth (Guerrero et al., 2014). Despite the efforts that have been devoted to Se biofortification studies, several factors, such as the focus on hyperaccumulator plants, the use of high Se doses and the lack of a consistent strategy in the selection of Se source, have made it difficult to reach of a consensus towards a widespread methodology for Se biofortification (D'Amato et al., 2020).

Cereals, which are considered non-accumulators, have an average Se content of 0.01–0.55 mg kg^{-1} fresh weight (Hawkesford & Zhao, 2007). Nevertheless, Se concentrations in wheat grown in seleniferous soils can be as high as 30 mg kg^{-1} dry weight (DW) (Lyons et al., 2005; Whanger, 2002), and exceptionally up to 62 mg kg^{-1} DW, in South Dakota, USA (Cubadda et al., 2010), and up to 387 mg kg^{-1} DW in Punjab, India (Eiche et al., 2015). Indeed, wheat has the highest Se accumulation capacity among cereals (Raina et al., 2021) and can store considerably high amounts of Se when enriched without a significant decrease in yield due to toxicity (Wang, Ali, et al., 2020). Since wheat is the second most important food crop and is already a major dietary source of Se (Boldrin et al., 2016), wheat is a good candidate for producing Se-biofortified food. Furthermore, wheat is one of the most produced and consumed cereals worldwide (Enghiad et al., 2017), and it is already one of the main sources of nutrients in diets (Hussain et al., 2010). In addition, the Se-biofortification of wheat has already been proven to be retained in the production of enriched wheat-based foods such as flour and bread (Hart et al., 2011), pasta (Poblaciones et al., 2014), and soup (Bañuelos et al., 2022).

Regarding the metabolization of Se in humans, since different chemical species have different bioavailability and toxicity, their chemical form, rather than the amount consumed, determines the final Se status and, ultimately, the associated nutritional and health benefits (Rayman et al., 2008). In general, inorganic Se species, such as selenite (Se(IV)) and selenate (Se(VI)) ions, are more toxic than their organic counterparts (Rayman et al., 2008). In addition, they have low bioaccessibility (e.g., Se(IV)) and low bioavailability (e.g., Se(VI)) (Fairweather-Tait et al., 2010). Oppositely, organic Se is more bioavailable and bioactive, as more than 90% of Se consumed in the form of selenoamino acids is absorbed by the body, resulting in an effective increase of blood Se levels and GPx enzymatic activity (Fairweather-Tait et al., 2010). Inorganic Se species are the predominant form of Se found in the environment. Selenate is more soluble and less absorbed and, consequently, more bioavailable for plant uptake (Yu et al., 2019), whereas selenite is immobilized by adsorption on iron and aluminum oxides and soil organic matter (Li et al., 2017). Thus, selenate is the main species present in agricultural soils (Raina et al., 2021), and it is usually the choice for agronomic fortification through Se-enriched fertilizers over selenite and the much more expensive organic forms (Alfthan et al., 2015).

Crop plants can take up selenite and selenate species through active membrane transporters in roots and leaves, but the two inorganic ions have distinct behavior. They follow different transport pathways (Raina et al., 2021; Zayed et al., 1998), which may influence the distribution and accumulation through the plant organs and the Se species formed. Plants can metabolize these inorganic ions into organic Se species, such as selenomethionine (SeMet), selenocysteine (SeCys), selenocystine (SeCyst), methylselenocysteine (MeSeCys), gamma-glutamyl-methylselenocysteine, dimethylselenide, dimethyldiselenide and selenocystathionine (Freeman et al., 2006; Winkel et al., 2015; Xiao et al., 2020).

The main Se species in wheat are the amino acids SeMet, MeSeCys and SeCyst, together with unmetabolized inorganic Se(IV) and Se(VI) (Wang, Ali, et al., 2020). Elemental selenium (Se(0)) has also been reported to be present in wheat (Xiao et al., 2021). Although the literature regarding wheat biofortification with Se is extensive, most research has only focused on the total Se content of wheat grain (Broadley et al., 2010; Zou et al., 2019) and addressing the importance of Se speciation in wheat grain has only achieved partial results. SeMet is reported to be the most abundant species in wheat grain, with values generally over 70% of the total Se for both native and supplemented grains, regardless of the amount, chemical form and application method of the Se biofortification (Cubadda et al., 2010; Galinha et al., 2015). Selenate is also commonly quantified, with literature reporting values below 5% (Galinha et al., 2015; Warburton & Goenaga-Infante, 2007). An extremely limited number of studies have achieved a successful determination of the five Se species (Di et al., 2023; Eiche et al., 2015; Hart et al., 2011; Wang et al., 2022; Wang, Ali, et al., 2020; Xiao et al., 2021), and the present study is the first to quantify six species in wheat. Likewise, very few have used complementary techniques to confirm species identification and validate the applied method (Aureli et al., 2012; Warburton & Goenaga-Infante, 2007). The majority of the speciation studies have exclusively employed high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS), which, in some cases, resulted in an incomplete characterization of the

overall species due to the low stability of certain Se species after the required sample pre-treatment steps and incomplete recoveries of the methodologies used (Połatajko et al., 2005; Whanger, 2002). These drawbacks can be overcome by direct speciation techniques such as X-ray absorption spectroscopy (XAS) which allows Se speciation in solid form without requiring extraction and pre-treatment steps. In general, the use of direct speciation techniques and/or combined approaches for Se analysis in plants is scarce (el Mehdawi et al., 2014; Freeman et al., 2006; Pickering et al., 2000), and only a few have studied wheat plants (Wang et al., 2015; Xiao et al., 2020, 2021).

Thus, a multidisciplinary approach is necessary for unraveling the complete chemical state of Se in plants to comprehend the Se metabolism and ultimately address how Se accumulation in plants should be optimized to improve the specific nutritional value of Se-enriched food (Raina et al., 2021). In this context, the objective of the present work is to use the complementary speciation information obtained from both HPLC-ICP-MS and XAS techniques to quantify six selenium species: SeMet, MeSeCys and SeCyst, Se(IV), Se(VI) and Se(0), to understand the different Se pathways in wheat depending on the inorganic Se source supplied. This has been accomplished by studying the distribution of the Se species along the plant (roots, shoots, and grains) to completely characterize the output of the Se biofortification process, as well as the effects of this biofortification on the plant physiological growth, nutrient concentration, and hormonal homeostasis. This information is essential to develop the best strategy to improve the crop yield and the production of Se-enriched wheat-based functional food to tackle human Se deficiency.

2 | MATERIALS AND METHODS

2.1 | Wheat culture

Plants of common wheat (*Triticum aestivum* L. cv. Pinzón purchased from Semillas Fitó S.A.) were hydroponically grown. Seeds were germinated in moistened filter paper at room temperature for 5 days. Then, seedlings were transferred to opaque plastic containers filled with continuously aerated, modified ½ strength Hoagland's nutrient solution buffered with MES (2-(N-morpholino)ethanesulfonic acid, $C_6H_{13}NO_4S$) to maintain a stable pH of 6.0 (Guerrero et al., 2014).

The culture was carried out in a controlled-environment growth chamber with 18-24°C temperature, 60–70% humidity and 320 μE m^{-2} s^{-1} light intensity with a short photoperiod of 8 h light/16 h darkness during vegetative growth and a long photoperiod of 12–16 h light/8–12 h darkness for flowering induction and grain production.

Two weeks old plants (four per treatment), with three leaves unfolded, were exposed to sodium selenite, sodium selenate or a 1:1 mixture of both at 10 μM. A control treatment without Se was also included. The solution was renewed weekly over 12 weeks until senescence to maintain constant water, nutrients, and Se levels. The elemental concentrations and stability of the species in the nutrient solution were monitored by inductively coupled plasma-mass spectrometry (ICP-MS) and HPLC-ICP-MS, respectively. Two cultures were performed in two different years under the same conditions. The results were averaged since the two cultures did not present significant differences resulting in eight plants per treatment.

After harvesting, roots were washed with ice-cold $CaCl_2$ solution to remove the elements from the root apoplast and then rinsed with deionized water. The plants were divided into roots, shoots (including stems and leaves) and spikes, weighted and stored at -20°C until further processing.

2.2 | Plant physiological growth parameters

Plant material was oven dried at 45°C for 4 days until reaching stable weight. The physiological effects of the different Se forms on wheat plants were evaluated by the biomass produced in terms of the percentage of the dry weight of roots, shoots (stem and leaves) and grains of the treated plants compared with control ones. Moreover, the effect on grain yield was tracked as the number of spikes per plant, grains per spike and grain weight.

The accumulation of Se in roots, shoots and grain was determined as well as the Se translocation factor, which is calculated as the shoot Se concentration divided by the root Se concentration.

The significance of the results was assessed by an ANOVA statistical analysis with 95% confidence with the TIBCO Statistica software (StatSoft).

2.3 | Hormone analysis

The plant hormones (±)-jasmonic acid (JA), salicylic acid (SA), (+)-cis, trans-abscisic acid (ABA) and 3-Indoleacetic Acid (IAA) were analyzed by LC-ESI-MS/MS in multiple reaction monitoring mode (MRM). These phytohormones were extracted following the methods of Llugany et al. (2013) and Xiao et al. (2020). Briefly, 250 mg of frozen roots and shoots were ground in an ice-cold mortar and repeatedly extracted with 750 μl Methanol:2-Propanol: Acetic acid (20:79:1, v/v/v). The supernatant was recovered after centrifugation and lyophilized. Then, the obtained pellets were dissolved in 250 μl of methanol and filtered with a Spin-X centrifuge tube filter of 0.22 μm cellulose acetate (Costar, Corning Incorporated,).

Quantification was done using a standard addition calibration curve spiking control plant samples with standard solutions ranging from 50 to 1000 ppb for SA and 5 to 100 ppb for JA, ABA, and IAA. Deuterated hormones (±)-jasmonic acid-d6 (JA-d6), (+)-cis, trans-abscisic acid-d6 (ABA-d6) and Indole d5-acetic acid (IAA-d5) at 30 ppb and salicylic acid-d6 (SA-d6) at 300 ppb were used as internal standards in all the samples and standards measurements. All standards were purchased from (Sigma-Aldrich). Separation was done using an HPLC Agilent 1100 (Waldrom) on an Acquity UPLC BEH C18 2.1 × 100 mm ID, 1.7 μm column (Waters, USA) at 50°C with a constant flow rate of 0.8 ml min^{-1} and 10 μl injection. The elution was

performed with a gradient between 0.1% of formic acid in methanol and 0.1% of formic acid in Milli-Q water, as detailed in Xiao et al. (2020). Detection was carried out with an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord) using the Turbo Ionspray source in negative ion mode.

2.4 | Total selenium and mineral nutrient analysis

Ground dry plant material (50 mg) was acid digested in HP500 PFA vessels with 4 ml of HNO_3:H_2O_2 (3:1) at 180°C and 1.9 atm for 45 min in a microwave digestion system (Mars 5, CEM).

Determination of ^{78}Se, ^{24}Mg, ^{31}P, ^{55}Mn, ^{56}Fe, ^{64}Zn, ^{65}Cu, and ^{98}Mo concentrations was done by ICP-MS (X Series 2, Thermo Fisher Scientific) using ^{45}Sc, ^{69}Ga, ^{89}Y, and ^{115}In as internal standards.

The macronutrients S, Ca and K were measured by Induced Coupled Plasma Atomic Emission Spectroscopy at the "Scientific and Technological Centers of the University of Barcelona (CCiTUB)".

A certificate reference material (CRM), SELM-1 (NRC, Canada), consisting of a Se-enriched yeast in which Se is found as SeMet, was used to validate the sample digestion procedure and Se determination. The determined Se concentration of 1993 ± 39 mg kg^{-1} (mean ± SD with $n = 8$) agreed with the certified value of 2059 ± 64 mg kg^{-1} (Mester et al., 2006).

2.5 | Conventional selenium speciation

Selenium species were determined by HPLC-ICP-MS (^{78}Se elution). Previously, 50 mg of ground and dried plant material was extracted by enzymatic digestion with 10 mg of protease XIV and 5 ml of degassed $NH_4H_2PO_4$ (saturated with nitrogen to ensure oxygen depletion). The flask was sealed and placed in an incubator for 16 h at 37°C, in darkness and with continuous stirring. Afterwards, samples were cooled down with an ice bath, filtered, maintained at 4°C, and analyzed within 4 h.

A PRP-X100 (Hamilton) strong anion exchange column, 250 × 4.1 mm, with a stationary phase of 10 µm particle diameter, was used for analysis at 20°C of 100 µl of sample, injected with an HPLC Spectra system (Thermo Fisher Scientific). The mobile phase was ammonium citrate with 2% MeOH at pH 5.0. A gradient elution was performed from 5 to 15 mM of ammonium citrate, as reported in Table S1. Peak identification and quantification were performed by external calibration and confirmed by spiking with commercial standards of sodium selenite, sodium selenate, selenomethionine, methylselenocysteine hydrochloride and selenocystine and using ^{115}In as an internal standard. A standard for selenomethionine oxide was prepared by SeMet oxidation with 30 µl of H_2O_2, and a standard for selenomethionine selenone with 1000 µl of H_2O_2.

Additionally, the SELM-1 CRM was used for the assessment of the method as well as the extraction efficiency and chromatographic recovery.

2.6 | Direct selenium speciation

X-ray absorption spectroscopy (XAS) provides element-selective chemical speciation information without the need for any sample pretreatment, thus avoiding issues due to incomplete recoveries and/or reactivity of the species. XAS measurements at Se K-edge were performed at the BM25A SpLine beamline of the European Synchrotron Radiation Facility (ESRF) using a Si(111) double-crystal monochromator. Energy calibration was done with Se(0) to 12,658 eV (Ravel et al., 2005). For the measurements, ground and dried material from roots, shoots or grains were pressed into pellets. The experiment was carried out at liquid nitrogen temperature to minimize radiation damage. The fluorescence signal was collected by a 13-element Si(Li) solid-state detector (e2V Scientific Instruments). Reference samples were prepared from pure Se standards of SeMet, SeCyst, MeSeCys, Se(IV), Se(VI), and Se(0) and measured in transmission mode using gas ionization chambers. Data reduction and normalization and subsequent linear combination fitting (LCF) analysis were performed with the Athena program of the Demeter software package (Ravel et al., 2005).

3 | RESULTS

3.1 | Selenium effect on plant development

The physiological effects of Se exposure on wheat plants are shown in Figure 1 and Table 1. There is a significant reduction in plant biomass (43% shoots, 55% grain) in selenite-treated plants compared control. Selenate and mixture treatments did not result in any statistically significant change. Selenite treatment did not alter the number of stems, but it reduced the number of stems that were able to generate a spike compared with the control plants. Additionally, the number of grains produced in each spike was not significantly affected, although the average weight of each single grain is significantly lower with respect to the control or the other treatments (Figure 1B). Consequently, 10 µM selenite does not reduce the kernel formation in the spike but impairs the kernel development and ripening to achieve a higher weight. On the other hand, selenate and mixture treatments did not affect wheat yield.

3.2 | Selenium effect on phytohormones

Plant hormones JA, SA, IAA, and ABA are known to be implicated in signaling pathways that respond to abiotic stresses (Wang, Song, et al., 2020). They were analyzed, and the results are shown in Figure 2. The JA concentration was not significantly affected in roots or shoots by the addition of any form of selenium compared with control, but shoots treated with selenite or with the mixture treatment had significantly higher JA than the selenate treated plants. Salicylic acid tended to decrease after selenite and selenate treatments, with a significant reduction in the roots of plants treated with selenate as

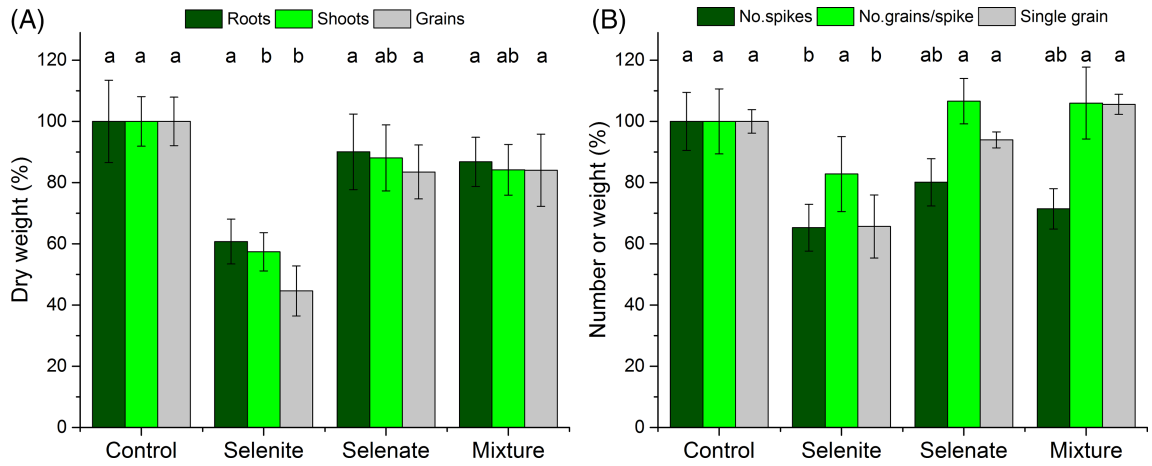

FIGURE 1 (A) Roots, shoots, and grains dry weight for each Se treatment, and (B) number of spikes, number of grains per spike and average dry weight of a single grain for each Se treatment expressed as relative percentage respect to the control plants (represented as mean ± SE, $n = 8$). Letters indicate significance ($p < 0.05$) between different treatments.

TABLE 1 Roots, shoots, and grains dry weight (g DW), number of spikes, number of grains per spike, and average dry weight of a single grain (mg DW) for each Se treatment

	Control	Selenite	Selenate	Mixture
Roots DW	1.5 ± 0.3 (a)	0.9 ± 0.2 (a)	1.4 ± 0.3 (a)	1.3 ± 0.2 (a)
Shoots DW	22 ± 2 (a)	13 ± 1 (b)	20 ± 3 (ab)	19 ± 2 (ab)
Grains DW	6.1 ± 0.6 (a)	2.7 ± 0.5 (b)	5.2 ± 0.7 (a)	5.1 ± 0.8 (a)
No. of spikes	9.0 ± 1 (a)	6.1 ± 0.7 (b)	7.3 ± 0.6 (ab)	6.6 ± 0.6 (ab)
No. of grains per spike	23 ± 3 (a)	19 ± 3 (a)	25 ± 2 (a)	24 ± 3 (a)
Single grain DW	30 ± 1 (a)	20 ± 3 (b)	28 ± 0.8 (a)	32 ± 0.9 (a)

Note: Results are shown as mean ± SE with $n = 8$. Letters indicate significance ($p < 0.05$) between different treatments.

well as in the shoots of plants treated with selenite. The 3-indoleacetic acid level tended to decrease with selenium exposure, and it was significantly reduced in the roots of both selenite and selenate plants, as well as the shoots of selenite and mixture treatments. Abscisic acid was not significantly affected by any of the treatments.

3.3 | Selenium uptake and distribution

The Se concentration in the roots, shoots, and grain is reported in Figure 3. The Se concentration in treated plants greatly differs from control plants, which demonstrates the successful uptake and accumulation of Se by wheat. The results reveal that the distribution of Se is not homogeneous through the different plant parts (roots, shoots, and grain) and depends on the form of Se present in the substrate. In selenite-treated plants, Se was highly accumulated in roots (66% of the total Se), and very little was translocated to shoots (8% in stems and leaves, 26% in grains). In contrast, in selenate treatments, only 18% of Se was accumulated in roots, whereas 46% was translocated to shoots and 35% to grains. Coherently, the mixture enrichment had an intermediate behavior, approximately 61% of the Se was found in roots, 15% in shoots, and 23% reached the grain. The translocation factor was lower than 1 for selenite (0.11 ± 0.01) and mixture (0.26 ± 0.04) treatments and higher than 1 under selenate exposure (2.57 ± 0.35). Therefore, the translocation to shoots was favored over root accumulation only on selenate treatments.

The Se concentration in roots was statistically different among the three treatments, being the highest in selenite biofortification. Shoots of plants treated with selenate had a significantly higher level of Se than shoots from selenite and mixture treatments. However, despite the difference in the translocation from root to shoot between selenite and selenate, the final Se concentration in grain was not statistically different between the two treatments. Only the mixture treatment resulted in a significantly lower selenium amount in grains (95 ± 11 mg Se kg^{-1} DW), in comparison with selenite (149 ± 29 mg Se kg^{-1} DW) and selenate (145 ± 8 mg Se kg^{-1} DW), which indicates that the simultaneous exposure to the two species does not result in an additive effect.

3.4 | Mineral nutrient analysis

The effects of Se over the assimilation of mineral macronutrients (S, P, K, Ca, Mg) and micronutrients (Fe, Mn, Cu, Mo, Zn) by wheat was studied through their quantification in roots, shoots, and grain, and are represented in Figure 4 (for S and P) and Figure S1 (for K, Ca, Mg,

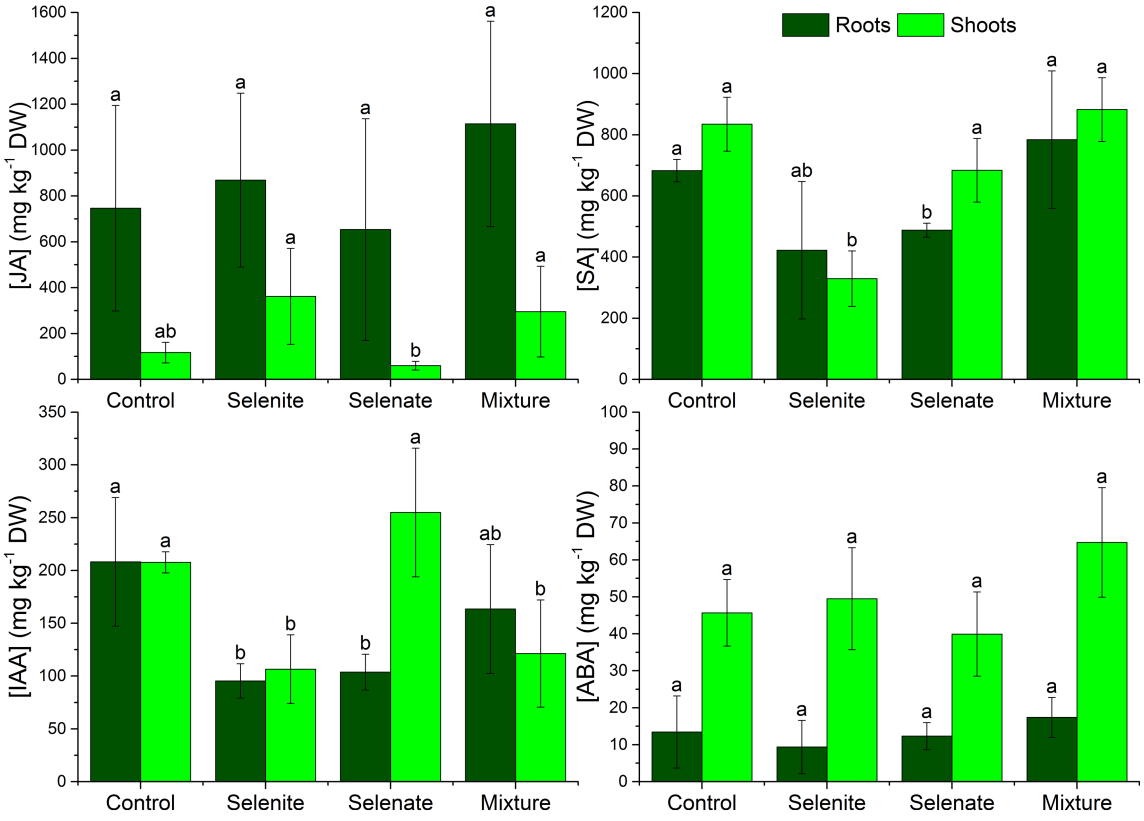

FIGURE 2 Phytohormone concentration in mg kg^{-1} DW in roots and shoots for jasmonic acid (JA), salicylic acid (SA), 3-indoleacetic acid (IAA) and abscisic acid (ABA), represented as mean ± SD ($n = 3$). Letters indicate significance ($p < 0.05$) between different treatments.

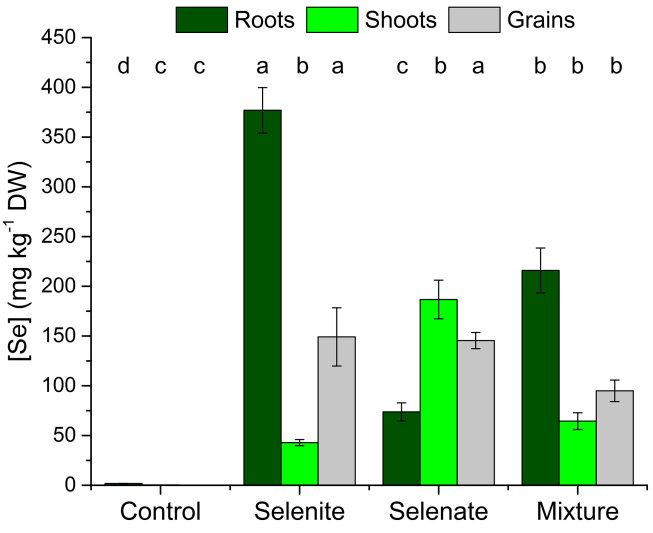

FIGURE 3 Selenium concentration in roots, shoots, and grains (mg Se kg^{-1} DW) represented as mean ± SD ($n = 8$). Letters indicate significance ($p < 0.05$) between treatments.

Fe, Mn, Cu, Mo, Zn). Selenite and selenate are known to be taken up and transported via sulfur and phosphorus transporters, and consequentially, those elements were the most affected by the selenium exposure. Particularly, the sulfur concentration was mainly altered with the selenate treatment, which caused S levels to be significantly lowered in the roots, but significantly increased in the shoots. The phosphorus concentration was significantly reduced in the roots of all selenium treatments, while no significant effects were seen in shoots and grains compared with control plants.

In addition, selenite treatment resulted in higher shoot and grain K concentration and a lower root Ca amount than the control treatment. Selenite exposure did not modify the levels of Mg in wheat tissues. Selenate treatment did not significantly change K concentration but resulted in Ca levels that were lower in roots and higher in shoots compared with the control, whereas Mg levels were only higher in roots. The mixture treatment resulted in the same trends for K and Ca as observed for the selenate treatment, with no significant effects in K and lower concentration of Ca in root and higher in shoots. Mg concentration was not significantly modified compared with the control.

No significant effects were seen in Fe, Mn, Zn, Cu, and Mo concentrations for the selenium-treated plants relative to the control.

3.5 | Indirect speciation

The metabolization of the Se species was characterized by tandem HPLC-ICP-MS after enzymatic digestion to achieve a deeper understanding of the transformation of Se in wheat. As shown in Figure 5, the five major species present in wheat tissues were identified to be SeMet, MeSeCys, SeCyst, Se(IV), and Se(VI). Their relative abundance

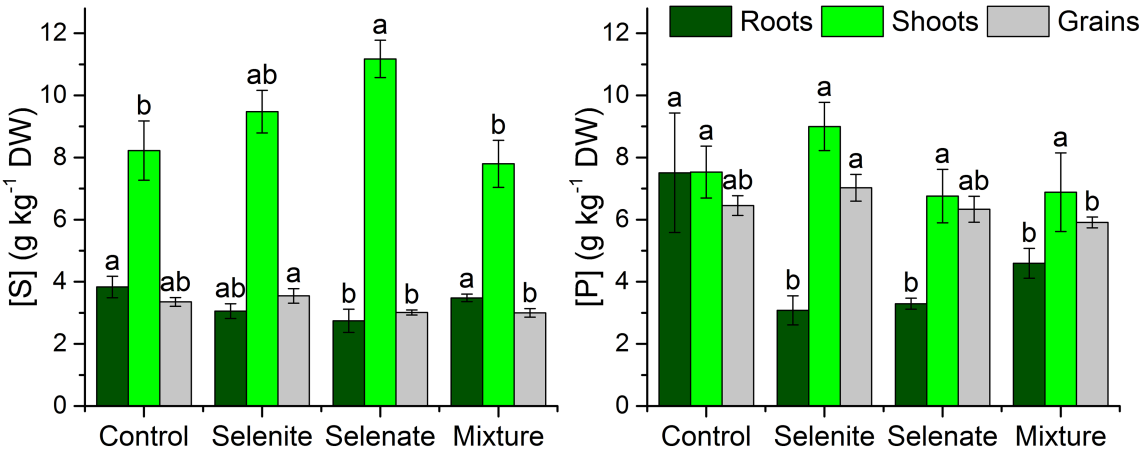

FIGURE 4 Sulfur and phosphorus concentration in roots, shoots, and grains (g kg^{-1} DW) represented as mean ± SD ($n = 8$). Letters indicate significance ($p < 0.05$) between treatments.

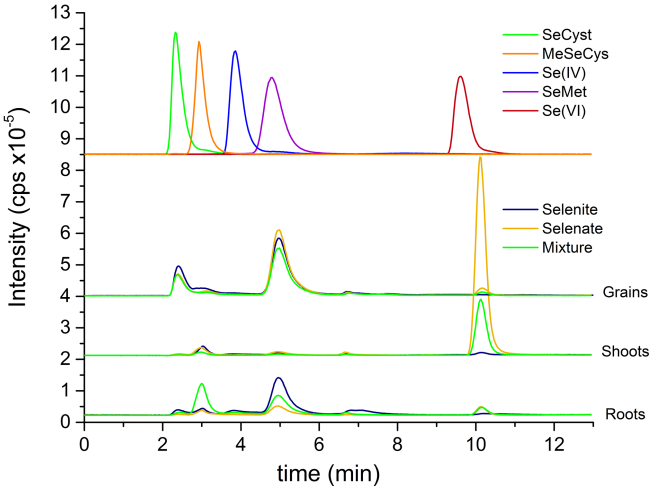

FIGURE 5 Chromatograms of the standard selenium species: selenocystine (SeCyst), methylselenocysteine (MeSeCys), selenomethionine (SeMet), sodium selenite (Se(IV)), and sodium selenate (Se(VI)) (top), and chromatograms of roots, shoots and grains of wheat samples enriched with selenite, selenate and mixture of both species (bottom), using the optimized gradient method of Table S1. The chromatograms have been shifted vertically for the sake of comparison.

was dependent on the organ analyzed and the growing conditions. Additionally, a few minor unknown peaks were also detected, which represent 2%–15% of the total area.

Selenoamino acid quantification by HPLC-ICP-MS requires careful attention since sample pre-treatment may promote the oxidation of SeMet to selenomethionine selenoxide. This yields a peak in the chromatogram close to the void-volume and to the SeCyst peak, which hampers an accurate determination (Cubadda et al., 2010; Galinha et al., 2015). Thorough care during the enzymatic digestion has been shown to minimize the SeMet oxidation below 4 ± 2% in the pure SeMet sample SELM-1 CRM. The extraction efficiency varied among the different tissues (50 ± 11% roots, 118 ± 34% shoots, and 115 ± 8% grain) but not among the enrichment treatments (Figure S2). The employed methodology also achieved an extraction efficiency of 71.3 ± 0.9% from SELM-1 CRM. These values lie within the typical 70%–90% range for both SeMet and enzymatic digestions in general (Aborode et al., 2015; Maher et al., 2012).

Shoots and grains show excellent extraction efficiencies, and consequently, Se is mainly found in the form of species that are readily soluble and extractable by mild extractions or by peptide bond cleavage. On the contrary, the roots show very poor extractions, ~50%, which could be explained by incomplete digestions due to the presence of Se in forms not completely digestible with the enzymatic protocol or insoluble, such as Se(0) (Aborode et al., 2015; Połatajko et al., 2005).

On the other hand, the average chromatographic recovery for all tissues and treatments was 54 ± 20%, while the recovery for SELM-1 was 73 ± 3% (in agreement with the values reported for both plants and yeast [Połatajko et al., 2005; Maher et al., 2012]). The low recovery in plant tissues might be due to poor solubilization of selenopeptides and non-peptidic high molecular weight compounds or to non-specific interactions of Se species, resulting in forms that do not give a well-defined peak or elute as a continuum during the chromatography (Połatajko et al., 2005). Consequently, due to limited extraction efficiency for roots and incomplete chromatographic recoveries, only a fraction of the Se species in wheat tissues is quantified by HPLC-ICP-MS, but this technique provides a preliminary insight into the selenium metabolism that occurs in wheat.

Regarding roots, chromatographic results from the extract always showed SeMet as the most abundant species, as shown in Table 2 and Figure 5. Selenite-enriched roots contained SeMet as 64 ± 5% of the total Se. The remaining inorganic Se was below the LOQ, but still, it was detected that a small part of the Se was oxidized to selenate, indicating a relatively oxidant environment in wheat roots. Oppositely, selenate-enriched roots had only 43 ± 7% of SeMet, with 27 ± 6% of Se remaining as selenate. However, very little selenite was detected. Consistently, in roots enriched by a Se mixture, selenite had been completely metabolized, but 10 ± 3% of selenate remained in the

TABLE 2 Selenium speciation determined by HPLC-ICP-MS in wheat roots, shoots, and grains enriched with selenite, selenate, and mixture of the species

		Concentration (mg Se kg^{-1} DW)				
		SeCyst	MeSeCys	Se (IV)	SeMet	Se (VI)
Roots	Selenite	<LOQ	4 ± 3	4 ± 3	29 ± 6	<LOQ
	Selenate	<LOQ	4 ± 4	<LOQ	11 ± 2	6.8 ± 0.9
	Mixture	<LOQ	21 ± 2	<LOQ	22 ± 6	5.2 ± 0.9
Shoots	Selenite	<LOQ	3.1 ± 0.7	<LOQ	3 ± 1	<LOQ
	Selenate	<LOQ	3 ± 2	<LOQ	6 ± 1	156 ± 21
	Mixture	<LOQ	<LOQ	<LOQ	3.6 ± 0.8	41 ± 7
Grains	Selenite	23 ± 15	3 ± 2	<LOQ	53 ± 9	<LOQ
	Selenate	15 ± 1	1.9 ± 0.2	<LOQ	73 ± 7	5.7 ± 0.6
	Mixture	11 ± 2	1.4 ± 0.4	<LOQ	50 ± 3	2.7 ± 0.4

Note: Results shown as mean ± SD of the selenium determination with $n = 4$.

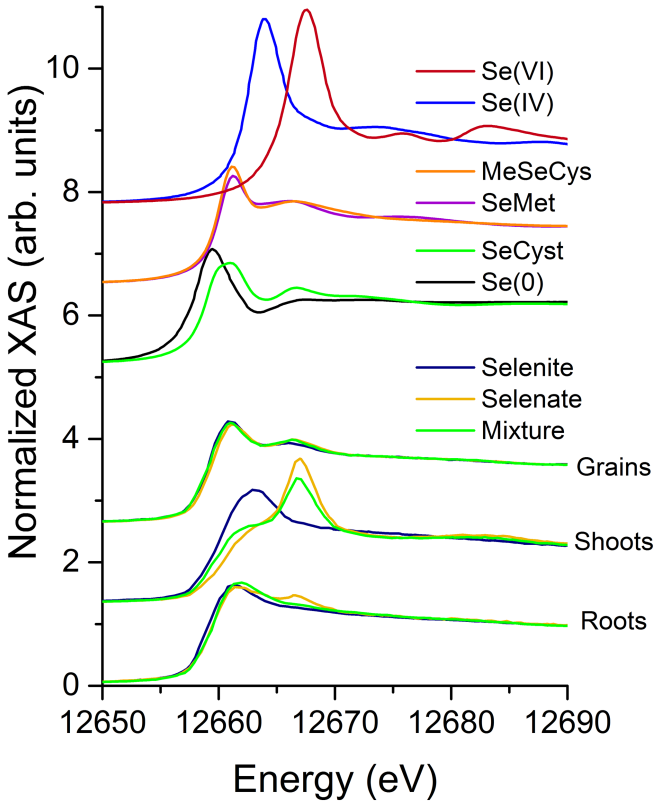

FIGURE 6 Normalized Se K-edge XANES spectra of roots, shoots and grains enriched with selenite, selenate and mixture treatments grouped by tissue type and XANES spectra of the reference compounds: Elemental selenium (Se(0)), selenocystine (SeCyst), methylselenocysteine (MeSeCys), selenomethionine (SeMet), sodium selenite (Se(IV)) and sodium selenate (Se(VI)). The spectra have been shifted vertically for the sake of comparison.

roots. In addition, SeMet accounted for 39 ± 7% of Se, but there was a peak that comprised 40 ± 7% of the total Se in Table 2, which coeluted with the standard of MeSeCys, but it is most probably a by-product of SeMet since there was interconversion between the two species with time and the addition of a reducing agent minimized its formation. The retention time of the peak matched that of selenomethionine selenone, which is formed by further oxidation of SeMet (Vonderheide et al., 2002), but the identification should be confirmed by complementary techniques such as LC–MS.

Regarding shoots, they presented a minimum accumulation of organic Se, as shown in Table 2 and Figure 5. The shoots enriched with selenite showed very little concentration of Se. All species were detected, but only SeMet and MeSeCys were above the limit of quantification. In contrast, selenate-enriched shoots showed an outstanding selenate accumulation, 92 ± 3%. The shoots treated with the mixture also presented a predominant accumulation of selenate, 81 ± 7%.

In grains, SeMet was the predominant species in all the enrichment treatments, followed by a significant amount of SeCyst (see Table 2 and Figure 5). Considering a relative concentration, the selenite-enriched grain had 25 ± 12% SeCyst versus 64 ± 15% SeMet, the selenate-enriched grain 15 ± 2% SeCyst and 73 ± 3% SeMet, and the mixture-enriched grain 16 ± 3% SeCyst and 73 ± 3% SeMet. Therefore, the different treatments resulted in modified proportions of selenoamino acids in the grain, with selenite treatment having the highest tendency to accumulate selenium as SeCyst.

Furthermore, very little inorganic Se reaches wheat grains. Se(IV) is hardly detected in any of the three enrichment conditions, and only 5.8 ± 0.5% Se(VI) is present in the selenate-enriched grains and 4.0 ± 0.5% in the mixture treatment. Consequently, despite all differences in behavior between selenite and selenate in roots and shoots, the differences in grain are somewhat smaller between the three biofortification treatments, with the main difference being a possible divergence in the proportion of SeCyst:SeMet found in grain.

3.6 | Direct speciation

As shown in Figure 6 for the spectra of the Se references, the XAS spectral profile reveals the chemical state of Se. The position of the absorption edge (E_0) is influenced by the electron density around the Se atom and reflects its oxidation state (roughly, higher E_0 for higher oxidation state, see Table 3). Thus, inorganic species such as Se(IV), Se(VI), and Se(0) can be differentiated by their relative position in energy. On the other hand, SeMet and MeSeCys selenoamino acids

have similar spectra due to the similar coordination of the Se atom, C-Se-C. Therefore, to account for this uncertainty in their identification, they have been grouped as C-Se-C compounds. However, the spectral profile of SeCyst (C-Se-Se-C structure) is markedly different from that of C-Se-C compounds. E_0 is found at significantly lower energies, and the white-line (first resonance after the edge) is significantly broader (Xiao et al., 2020).

The spectra of roots, shoots and grains of wheat plants revealed spectral differences depending on the organ and the biofortification treatment used (Figure 6). An LCF analysis using the reference spectra allowed the determination of the species contributing to each sample spectrum. Results are displayed in Table 4 (fits can be found in Figures S3–S5).

Roots had a similar spectral profile, and their white-line is characteristic of the C-Se-C organic species (selenite 82 ± 6%; selenate 94 ± 5%; and mixture 99 ± 7% of C-Se-C). In the case of selenite treatment, the presence of insoluble elemental Se (18 ± 8%) can explain the lower E_0 and the very low extraction efficiency after enzymatic digestion. A much smaller amount was also present in the roots treated with the mixture. On the other hand, the spectra of selenate-enriched roots showed a second subtle shoulder that can be assigned to a small presence of Se(IV) and a slightly more marked third characteristic feature due to the presence of Se(VI).

TABLE 3 Absorption edge position (E_0) determined from the spectra of selenium references and plant samples

Standard	E_0 (eV)	Sample	E_0 (eV)
Se(0)	12658.0	Roots selenite	12658.9
SeCyst	12658.5	Roots selenate	12659.2
MeSeCys	12659.3	Roots mixture	12659.5
SeMet	12659.8	Shoots selenite	12659.8
Se(IV)	12662.4	Shoots selenate	12660.5
Se(VI)	12666.0	Shoots mixture	12660.4
		Grains selenite	12659.2
		Grains selenate	12659.4
		Grains mixture	12659.3

In shoots, selenite-treated plants showed a single broad feature attributed mainly to C-Se-C organic species (81 ± 14%) and some non-metabolized selenite (19 ± 2%). The other treatments, selenate and mixture, showed a predominance of inorganic species, mostly Se(VI) (selenate 47 ± 1%; mixture 32.1 ± 0.8%) and also some Se(IV) (selenate 19 ± 2%; mixture 12 ± 2%) while the organic species of C-Se-C remain an important contribution (selenate 35 ± 15%; mixture 56 ± 11%). These results show an almost complete metabolization in the case of selenite enrichment and a limited transformation in the case of selenate.

In grains, the differences among treatments were smaller than in roots and shoots, and their spectra resembled the spectral profile of the selenoamino acids. The position of E_0 and the intensity of the second feature are the main differences among treatments. This suggests that all three enrichment conditions resulted in grains containing C-Se-C (selenite 57 ± 6%; selenate 74 ± 5%; mixture 62 ± 6%) and C-Se-Se-C species (selenite 44 ± 2%; selenate 25 ± 2%; mixture 38 ± 2%), being the remaining inorganic content very low or negligible. In general, the C-Se-C species are assumed to be the main or only component of Se in wheat grain (Galinha et al., 2015). However, although the selenate biofortified grains had the highest C-Se-C content, there was still a relevant amount of C-Se-Se-C. In fact, the proportion of C-Se-C/C-Se-Se-C amino acids found in wheat grain depends on the species used in the biofortification and increases according to the following trend: selenite < mixture < selenate. This can be attributed to the different behavior of each Se species in the wheat plant.

Although the shoots for all treatments accumulated a significant amount of inorganic Se, it is important to note that little or no inorganic Se is translocated to the grain. Only 1.5 ± 0.3% Se(VI) was detected for the selenate treatment. Therefore, only organic Se is effectively translocated to grains.

4 | DISCUSSION

The aim was to investigate the effect of inorganic Se species in wheat plants. Our results show that the biofortification of wheat with 10 μM

TABLE 4 Results from linear combination fitting analysis of Se K-edge XAS

			Relative concentration (%)				
		R-factor	Se (0)	C-Se-Se-C	C-Se-C	Se (IV)	Se (VI)
	Selenite	0.002	18 ± 8	n.d.	82 ± 6	n.d.	n.d.
Roots	Selenate	0.001	n.d.	n.d.	94 ± 5	4.4 ± 0.7	1.4 ± 0.3
	Mixture	0.002	1 ± 1	n.d.	99 ± 7	n.d.	n.d.
	Selenite	0.009	n.d.	n.d.	81 ± 14	19 ± 2	n.d.
Shoots	Selenate	0.009	n.d.	n.d.	35 ± 15	19 ± 2	47 ± 1
	Mixture	0.004	n.d.	n.d.	56 ± 11	12 ± 2	32.1 ± 0.8
	Selenite	0.003	n.d.	44 ± 2	57 ± 6	n.d.	n.d.
Grains	Selenate	0.001	n.d.	25 ± 2	74 ± 5	n.d.	1.5 ± 0.3
	Mixture	0.002	n.d.	38 ± 2	62 ± 6	n.d.	n.d.

Note: The weight of each component is expressed as a percentage of the total. R-factor is a measure of the mean square sum of the misfit at each data point which indicates the goodness of fit.

of selenium successfully improves Se content in wheat grain, leading to Se concentrations between 90 and 150 mg kg^{-1} DW. Selenium biofortification may also have a detrimental physiological effect on wheat development and grain yield, depending primarily on the fortifying inorganic Se species. Although Se enrichment has been performed at concentration levels that do not show a significant effect on wheat seedlings in short-term hydroponic studies (Boldrin et al., 2016; Guerrero et al., 2014; Li et al., 2008), long-term exposure to 10 μM Se in the form of selenite resulted in poor crop performance (Figure 1). On the other hand, the use of selenate or a mixture of both does not hinder grain development. Consequently, the selection of Se species for biofortification is relevant, with only selenite being a less suitable option.

The decrease in the plant parameters under selenite treatment can be understood in terms of Se biochemistry. Selenite accumulates in roots up to 377 ± 23 mg Se kg^{-1} DW, producing Se-induced toxicity to the plant, which inhibits the development of roots and shoots (Kolbert et al., 2016; Terry et al., 2000), and impairs the production and weight of the seeds (Prins et al., 2011). This behavior is in agreement with previous works that reported the impact of Se at lower concentrations for selenite in non-accumulators such as wheat (Guerrero et al., 2014), lettuce (Hawrylak-Nowak, 2013) and cucumber (Hawrylak-Nowak et al., 2015). The distinctive toxic effects caused by the two inorganic Se species suggest differentiated mechanisms in the Se uptake and metabolism, as the concentration and distribution of Se throughout the organs differ depending on the treatment (Figure 3). This can be attributed to the separate pathways for selenite and selenate assimilation in the roots (Zayed et al., 1998). Selenate is taken up actively via sulfate transporters *SULTR1;1* and *SULTR1;2* in the root plasma membrane, competing with sulfate (Gupta & Gupta, 2017; Raina et al., 2021). In contrast, as described for rice, selenite uptake is mediated by the phosphate transporters *OsPT2* and *OsPT8* (Zhang et al., 2014; Zhou et al., 2020), and by the silicon transporter *OsNIP2;1* (Zhao et al., 2010).

Such Se-induced toxicity occurs through complex mechanisms: (i) Se interference in the sulfur metabolism (Hawrylak-Nowak, 2013), where Se non-specifically replaces sulfur in amino acids, modifying the structure and function of proteins (Kolbert et al., 2016); (ii) increased oxidative stress, since plants have less capacity to eliminate the free oxygen radicals produced, which translates in the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, and reactive nitrogen species, such as nitric oxide and peroxynitrite, which can cause damage to the cellular structures and biomolecules (Freeman et al., 2010; Kolbert et al., 2016); (iii) intrusion of Se in the cellular energy production pathway, affecting the activity of the photosynthetic system and the levels of photosynthetic pigments (Hawrylak-Nowak et al., 2015; Mostofa et al., 2017); and (iv) Se interference in the nutritional status of plants, affecting the content of essential elements, reducing sugars, proteins and antioxidant compounds and affecting the homeostasis of several phytohormones involved in the regulation of growth and response to stress (Kolbert et al., 2016; Mostofa et al., 2017; van Hoewyk et al., 2008). These toxicity effects on wheat plants treated with Se can alter the concentrations of mineral nutrients and phytohormones. Regarding nutrients, our results show a modification in the levels of S and P. The competition with Se for the same sulfate and phosphate transporters caused a tendency to decrease S and P concentrations in roots exposed to Se, while other mineral nutrients were less affected. It is well known that selenite affects the uptake and accumulation of P, and it is not so frequent to find this effect also for selenate, but in previous work by our group with the same cultivar of wheat (Guerrero et al., 2014) the same reduction in the root levels of P but not in that of the shoots was observed. In addition, Zafeiriou et al. (2022) found that selenate reduced P levels in lettuce plants, but the authors attributed this to selenate toxicity. In our study, the plants did not suffer acute toxicity and no significant differences in biomass between control plants and those treated with selenate were observed. Another study in wheat (Zhang et al., 2017) observed that variable amounts of phosphorus affected the selenate uptake from soil. Those authors concluded that the changes in soil pH with the phosphorus application led to higher Se mobilization. This effect is excluded as our study has been done in a hydroponic solution with a buffered pH. That study also states that current literature does not explain how P affects Se uptake and translocation in wheat. However, it is well known that the inhibition of sulfate uptake by selenate in sulfate transporters is much stronger than that of phosphate by selenite in phosphate transporters due to the greater chemical differences in the latter molecules (Hopper & Parker, 1999). Thus, it is possible to hypothesize that phosphate transporters are less specific and can also transport small amounts of selenium as selenate, but this effect should be studied in further detail. In the case of plant hormones, they play a key role in growth and development through their participation in signaling pathways. JA, SA, IAA, and ABA are known to respond to abiotic stress such as salinity, heavy metals exposure, cold, drought, light, and other environmental stress factors (Wang, Song, et al., 2020).

When plants are exposed to high concentrations of Se, their response could be comparable to that caused by heavy metal stress. Although phytohormone responses are complex due to their dependent regulatory responses, with both synergistic and antagonistic regulations, in general, JA and ABA levels tend to increase, while SA and IAA levels decrease with abiotic stress (Wang, Song, et al., 2020). The toxicity response of wheat to Se exposure found in the present work agrees with this tendency. The growth hormone IAA decreased with Se exposure, especially in selenite, for which both roots and shoots were affected, which is consistent with the lower biomass observed in selenite-treated plants. Plants exposed to selenite, either alone or with selenate in the mixture treatment, had higher levels of JA in shoots than the selenate-treated plants or control plants. Jasmonic is known to enhance S uptake to prevent S replacement by Se in proteins and other S-containing compounds (Tamaoki et al., 2008), which is also consistent with the higher amounts of S in roots and grains of plants under selenite and mixture treatments compared with selenate. The competition between selenate and sulfate for the same transporters results in lower S levels in selenate-treated plants, which counteracts the positive effect that JA could have in this treatment, and thus the JA levels are not enhanced in the selenate treatment in

comparison to the selenite. On the contrary, ABA is related to responses to water deficiencies and low temperatures (Wang, Song, et al., 2020). The absence of significant effects for this hormone indicates that the toxicity suffered by plants is due to the Se exposure and excludes the effect of other stresses during plant growth. Furthermore, Se had previously been shown to reduce IAA content in the roots and shoots of wheat plants, especially after selenite treatment (Xiao et al., 2020). Whereas, regarding other plants, Se exposure resulted in an increase in JA in *Arabidopsis* (Tamaoki et al., 2008) and a lower SA in *Stanleya pinnata* and *Stanleya albescens* (Freeman et al., 2010).

Furthermore, the translocation from roots to shoots also varies (Dumont et al., 2006). Selenate is more easily transported to shoots than selenite or organic selenium forms (Hawrylak-Nowak et al., 2015; Terry et al., 2000). Translocation depends on the xylem loading rate, plant transpiration, physiological and environmental conditions (Gupta & Gupta, 2017), and the diffusion coefficient of the species. Selenate is readily transferred from the root epidermal cells to the xylem, and selenate-treated plants show higher Se concentration in xylem exudates than in selenite treatments (Yu et al., 2019). Thus, mobility through the xylem depends on the diffusion coefficient of the species in solution, with the diffusion coefficient of selenate being 2 to 3 orders of magnitude greater than that of selenite in a variety of media and conditions, and the coefficients of diffusion of organic Se species fall somewhere in between (Shen et al., 1997). Consequently, the translocation capacity in plants for the different species follows the trend: selenate > > selenoamino acids > selenite (Gupta & Gupta, 2017; Zayed et al., 1998), which is in accordance with the results of this study.

In addition to the different transport and translocation pathways for selenite and selenate, different genes and different loci have been shown to be involved in the tolerance mechanisms to each inorganic Se specie in *Arabidopsis* plants (Van Hoewyk et al., 2008; Zhang et al., 2006), also indicating a possible genetic factor in the distinct response of wheat to selenite and selenate.

Furthermore, the metabolism of Se species is also uneven. Selenate must be reduced to selenite before converting to selenide and subsequently to organic species (Hawkesford & Zhao, 2007; Winkel et al., 2015). In roots, the notable amount of unmetabolized selenate indicates that the reduction of selenate to selenite is a slow process, but the absence of selenite indicates that, once selenite is formed, its reduction to organic selenium occurs rapidly. These facts confirm that the selenate to selenite transformation is the rate-limiting step of Se metabolism in wheat (Terry et al., 2000; Wang et al., 2015; Zayed et al., 1998). In other words, selenite has a shorter chemical path to the final selenoamino acid moiety than selenate.

Coherently, selenate translocation to aerial parts occurs faster than its reduction, so selenate preferentially accumulates in shoots. A similar process occurs with sulfur. Sulfate is assimilated and reduced in chloroplasts, but when the concentration in the xylem is too high, it is also stored in the vacuoles of the leaf mesophyll cells (Hopper & Parker, 1999). Sulfate in the vacuoles is not metabolized, is innocuous to the plant, and is rarely remobilized (Hopper & Parker, 1999). The same phenomenon occurs with selenate, which accumulates unmetabolized in the shoots vacuoles and does not contribute to initiating a toxicity response (Hopper & Parker, 1999).

On the other hand, the quick reduction of selenite and its low translocation capacity results in a high Se accumulation in selenite-treated roots, 377 ± 23 mg Se kg^{-1} DW. This value is well above the toxicity threshold of 100 mg Se kg^{-1} of DW, generally set for non-accumulator plants (Gupta & Gupta, 2017). Even if wheat has a higher tolerance to Se accumulation compared with other cereals (Raina et al., 2021; Wang, Ali, et al., 2020), previous research has shown that only above 200 mg Se kg^{-1} DW a significant growth inhibition begins to occur and becomes critically toxic over 325 mg Se kg^{-1} DW (Cubadda et al., 2010). Therefore, the concentration of Se in selenite-treated roots exceeds these limits. Those high Se levels can interfere with plant homeostasis and cause Se-induced stress, triggering the production of ROS and generating phytotoxicity, justifying the decrease in plant development and crop yield discussed above for this treatment.

The Se concentrations in wheat tissues are certainly high for a non-accumulator like wheat. However, other hydroponic studies of wheat have reported comparable results. Li found between 5 and 45 mg Se kg^{-1} DW in roots only after 24 h of plant exposure to Se (Li et al., 2008). Guerrero reported up to 90 mg Se kg^{-1} DW after only 5 days of Se exposure in seedlings (Guerrero et al., 2014). Xiao found Se levels in roots and shoots within 25–250 mg Se kg^{-1} DW after only 2 weeks of Se exposure (Xiao et al., 2020), and 37–138 mg Se kg^{-1} DW in grains after 14 weeks of exposure (Xiao et al., 2021). Furthermore, in non-enriched naturally seleniferous soils from Punjab, India, wheat cultivation resulted in Se concentrations up to 146 mg Se kg^{-1} DW in vegetative tissues and 185 mg Se kg^{-1} DW in grains (Cubadda et al., 2010), and, a subsequent investigation reported 196 mg Se kg^{-1} DW in roots, 191 mg Se kg^{-1} DW in stems and 387 mg Se kg^{-1} DW in leaves (Eiche et al., 2015).

Regarding the chemical speciation shown in this work, the use of direct and indirect speciation techniques allowed the possibility of obtaining complementary results. This was essential to validate the speciation analysis performed by each method and, more importantly, to quantify six different species in wheat, which cannot be done by a single technique. The quantification achieved with HPLC-ICP-MS is only partial since it only accounts for small soluble compounds, and thus, elemental selenium cannot be determined. Otherwise, XAS does not have this limitation, and all the species could be identified, but the similar local atomic structure around Se of SeMet and MeSeCys makes their distinction difficult. In this sense, XAS allowed us to confirm the presence of Se(0) in the roots treated with selenite and the mixture. Elemental Se is present in wheat (Xiao et al., 2021) and the roots of other plants (Aborode et al., 2015; Valdez Barillas et al., 2012). Elemental Se is a plausible intermediate species when inorganic Se is reduced to selenide before further metabolization to organic Se (Winkel et al., 2015). Additionally, certain enzymes can also generate Se(0) and alanine from SeCys (Ellis & Salt, 2003), and microorganisms could also play a role in its production. Since Se(0) is biologically inactive (Hopper & Parker, 1999), the formation of this insoluble form when Se is supplied as selenite could be a mechanism to counteract the stress from the

excessive accumulation in roots. Concerning grains, all treatments resulted in Se mainly found as selenoamino acids SeMet, SeCyst and a small quantity of MeSeCys. The fact that the translocation of inorganic Se to the reproductive organs is minimal is advantageous to produce fortified food products since inorganic Se is more toxic than its organic counterparts (Rayman et al., 2008). These findings agree with previous studies on wheat, even though our conditions led to lower amounts of inorganic Se and a higher percentage of SeCyst (Di et al., 2023; Wang et al., 2022; Wang, Ali, et al., 2020).

Furthermore, the present study has shown that the enrichment conditions influence the selenomethionine/selenocystine proportion. The different mechanisms for selenite and selenate discussed above cause the diversity in the production of the specific selenoamino acids. Selenite treatments resulted in the accumulation of Se in the roots in high concentrations, causing a phytotoxicity response and the generation of ROS (Freeman et al., 2010). ROS species create an oxidizing environment in plant tissues (Mostofa et al., 2017) that enhances Se-Se bounds formation from selenol groups (Reich & Hondal, 2016), resulting in the oxidation of selenocysteine residues to selenocystine in the grain. In contrast, selenate stored in leaf vacuoles is stable and harmless to the plant (Hopper & Parker, 1999). Therefore, it does not promote an oxidizing environment, which results in methylated selenoamino acids in the grain in the form of C-Se-C, such as SeMet and MeSeCys. Alternatively, the characteristic selenoamino acid production can also be explained as a tolerance strategy. SeCys and SeMet can be non-specifically incorporated into proteins in place of cysteine and methionine (Freeman et al., 2006). The larger size, polarizability, and reactivity of Se (Reich & Hondal, 2016) lead to protein misfolding, which alters protein function and cellular biochemical reactions (Kolbert et al., 2016). This nonspecific incorporation of selenoamino acids into proteins might be a major cause of the harmful effect of Se on sensitive plants. The enhanced formation of SeCyst and MeSeCys, which cannot be incorporated into proteins, can be a defense mechanism of the plant to counteract the toxicity caused by selenite enrichment.

The difference in the selenoamino acid content depending on the enrichment conditions can be exploited to enhance the health benefits of the use of Se-biofortified wheat grains as a functional food. SeCyst, MeSeCys, and SeMet are metabolized differently by humans and, accordingly, contribute to disease prevention (Weekley & Harris, 2013). In particular, the opposite outcomes of the NPC and SELECT cancer trials have demonstrated the critical difference in the antineoplastic activity of the selenium compounds (Weekley & Harris, 2013). Both SeCyst and MeSeCys (Chen et al., 2019; Weekley & Harris, 2013) are more active species than SeMet, highlighting the importance of comprehensive Se speciation.

Our results demonstrate that it is possible to increase the amount of SeCyst in the grain by modifying the inorganic selenium species in the enrichment and, thus, substantially enhance the beneficial effects of consuming biofortified wheat.

AUTHOR CONTRIBUTIONS

Maria Angels Subirana contributed to the original idea, the research design, wheat cultivation and sample analysis, XAS data processing, manuscript writing and discussion of the results. Roberto Boada contributed to XAS data interpretation, manuscript writing and discussion of the results. Tingting Xiao and Mercè Llugany contributed to hormone and mineral nutrient analysis. Manuel Valiente and Mercè Llugany contributed to the original idea, research design and discussion of the results. All authors read and approved the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author, Mercè Llugany, and the first author, Maria Angels Subirana, upon reasonable request.

ORCID

Mercè Llugany https://orcid.org/0000-0002-9940-0383

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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