Co-application of Se and a biostimulant at different wheat growth stages: Influence on grain development.

Tingting Xiao[1], Roberto Boada[1], Mercè Llugany[2]*, Manuel Valiente[1]

[1]GTS-UAB Research Group, Department of Chemistry, Faculty of Science, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[2]Plant Physiology Group (BABVE), Faculty of Biosciences, Universitat Autonòma de Barcelona, 08193 Bellaterra, Spain

*Correspondence: merce.llugany@uab.cat, Tel: 3435811794

ABSTRACT

An appropriate selenium intake can be beneficial for human health. Se-biofortified food in Se-deficient regions is becoming an increasingly common practice but there are still issues to be addressed regarding the observed Se-induced toxicity to the plant. In this respect, plant biostimulants are used to enhance nutrition efficiency, abiotic stress tolerance and crop quality. In this work, the efficacy of a plant biostimulant to counteract the Se-induced stress in wheat plants is experimentally assessed. The co-application of different Se-biofortification treatments and the biostimulant at different growth stages (tillering or heading stage) was investigated. The use of micro focused X-ray spectroscopy allows us to confirm organic Se species to be the main Se species found in wheat grain and that the proportion of organic Se species is only slightly affected by the Se application stage. Our study proves that the biostimulant had a key role in the enhancement of both the amount of grains produced per spike and their dry biomass without hindering Se enrichment process, neither diminishing the Se concentration nor massively disrupting the Se species present. This information will be useful to minimize both plant toxicity and economic cost towards a more effective and plant healthy selenium supplementation.

Key words: Se speciation, XRF, XAS, Wheat, Grain, Plant biostimulant

1. INTRODUCTION

The importance of selenium (Se) for human health has been widely confirmed in several human nutrient studies (Ellis and Salt, 2003; Navarro-Alarcon and Cabrera-Vique, 2008; Thomson, 1998; Weekley et al., 2012). Se substitutes sulfur (S) in the amino acid groups forming antioxidant enzymes such as glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinase (IDD) which are important, among other things, for protecting against oxidative stress and for regulating the thyroid hormone metabolism. Currently, inadequate dietary Se intake affects up to 1 in 7 people globally with the associated risk of developing several chronic degenerative diseases (Fordyce, 2013; James et al., 1989; Rayman, 2000). To overcome this issue, Se supplementation has been extensively used (e.g. to control Keshan disease in China, and as adjunctive therapy in the treatment of Hashimoto's thyroiditis (Chen, 2012; Daniels, 1996; Toulis et al., 2010). Food derived from plants is a natural source of Se since plants can transform inorganic Se species present in soil into organic Se ones (e.g. seleno-amino acids) which are the desired form of Se for human diet. Thus, Se level in soil has usually a direct influence in the concentration of Se present in food and, subsequently, in the human body (Navarro-Alarcon and Cabrera-Vique, 2008). Since 1984, soil fertilization with Se has been applied in Finland to increase Se concentration of food in regions with Se-deficient soils (Varo et al., 1988). However, the presence of high concentration of Se in soil induces stress to the plant and may hamper its normal development (Guerrero et al., 2014). In order to overcome this issue, genetic engineering has been proposed as a strategy to enhance Se accumulation, volatilization and/or tolerance (Lüttge, 1962). However, this approach has serious potential risks since it might promote the presence of new allergens in food, (Buchanan, 2001) and it may promote the accumulation of other undesired heavy metals. Moreover, the rather elaborated procedures and challenges associated with the Se-enriched methodologies based on genetic engineering also need to be considered.

Alternatively, we propose to use a plant biostimulant, called Fyto-fitness (BIO Fitos, S.R.O., Czech Republic), based on hybrid heteropolyoxometalates (containing Mo,

B, Si, W and V) of Keggin structure mixed with humic acid, as anti-stressor to alleviate the Se-induced toxicity in the plant. Despite the fact that the application of anti-stressors is an increasing field of research in agriculture (Calvo et al., 2014), only few previous works have explored the possibility of applying a biostimulant to crops exposed to Se fertilizers. In this respect, Peng et al. (2001) reported that the use of fulvic acids as biostimulant has beneficial and antagonist effects depending on the dosage of selenite. However, the authors did not provide any information regarding the final Se concentration or the Se species present in the plants which is important to assess the health benefits of the Se-enrichment process.

In this work, we have studied the biostimulant effect on counteracting the Se-induced toxicity aiming to maintain the grain production yield, to minimize the Se-induced stress and to optimize the Se supplementation methodology. We have applied different Se treatments (selenite, selenate and a 1:1 mixture of both) together with the biostimulant at two growing stages, tillering stage or heading stage, until harvesting the grains once matured. We have determined the total Se concentration in grain by ICP-MS and the spatial distribution of Se and other relevant elements for the plant metabolism (e.g. Se, Ca, Zn) or for human nutrition by μXRF measurements. In addition, since determining the chemical state of Se is crucial to assess the health benefits of the biofortification procedure, μXANES spectra were collected at the most representative regions of the grain to get detailed information about the Se speciation. These measurements have allowed us to assess the possible modifications induced by the application of the plant biostimulant on the Se distribution and speciation in the wheat grain.

2. METHODOLOGY

2.1 Culture conditions

Wheat (*Triticum aestivum* L. cv. Pinzon) seeds (Fitó S.A., Spain) were germinated on moist filter paper for 5 days at 25 °C in the dark. Seedlings were precultured in continuously aerated ½ strength Hoagland's nutrient solution (Arnon and Hoagland, 1940) (3mM KNO_3, 2mM $Ca(NO_3)_2 \cdot 4H_2O$, 1mM KH_2PO_4, 0.5mM $MgSO_4 \cdot 7H_2O$, 60μM FeNa-EDTA, 2μM $MnCl_2 \cdot 4H_2O$, 3μM H_3BO_3, 0.1μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2μM $ZnSO_4 \cdot 7H_2O$, 1μM $CuSO_4 \cdot 5H_2O$) for two weeks before applying Se (12

plants per 6L pot). The pH of the solution was buffered at 6.0 with 2 mM MES (2-morpholinoethanesulphonic acid) and adjusted with KOH (2 M) (both from VWR, Spain). Plants were grown hydroponically in a controlled-environment growth chamber until mature with the following conditions: 8h day/16h night photoperiod with a light intensity of 320 $\mu Em^{-2}s^{-1}$.

2.2 Selenium and biostimulant treatments

Phyto-fitness (BIO Fitos S.R.O., Czech Republic) consists of an aqueous solution containing a mixture of hetero-polyanions (HPA), such as phosphomolybdate, silicotungstate, borovanadate, titanomolybdate and combinations thereof, esterified by humic acids. In addition, it also contains elemental iodine and micro / nano colloidal copper iodide. Both substances are responsible for the therapeutic effect against fungal, bacterial and viral infections, and urea is also present for a better absorption. Highest content of active substances in the used concentration is of 0.007% by weight.

In order to evaluate the effect of the plant biostimulant (Phyto-fitness) on the Se uptake and on the Se accumulation in the plant, plants were grown with (FA, foliar application) or absence (NB, no biostimulant) of the biostimulant. The foliar application of the biostimulant was done by spraying the product 100 times diluted in water on the leaves. Moreover, the plants were exposed to different Se treatments in the Hoagland solution: No Selenium (No Se); 10 µM selenite (Se(IV)) as Na_2SeO_3 (AMRESCO, USA), 10 µM selenate (Se(VI)) as Na_2SeO_4 (FLUKA, Spain) and a 1:1 v/v mixture of both Se treatment solutions (Se(MIX)). Hence, a total of 8 different treatments were applied.

In addition, with the aim of assessing both the Se-induced toxicity to the plant and minimizing the economic cost of Se supplementation, two batches of plants were grown and the treatments were applied at two different growing stages: from the tillering stage and from heading stage. In both cases, the treatments were maintained until the grain became mature. Afterwards, plants and grains were harvested and kept until further analysis. See the schematic diagram in Fig S1.

2.3 Total Se analysis

Powdered plant samples (n=4) were predigested overnight with HNO_3:H_2O_2 (7:3, v/v) (VWR, Spain) and then digested in hot block (SC154-54-Well Hot Block^{TM}) at 110 °C for 2 h. Mineral nutrient concentrations were analyzed by ICP-MS (PerkinElmer Optima 8300) and ICP-OES (PerkinElmer Nexton 350D). Blanks were included in each batch of samples for quality control.

2.4 Statistics

To check the reproducibility of the results, the entire experiment was repeated twice in different seasons; spring and summer. The results are presented as the mean (n=4) and the standard error (±SE) has been also included. All the data was checked for normality and data not normally distributed was log transformed. Afterwards, to assess the differences among treatments, two-way ANOVA followed by Fisher's LSD test ($P<0.05$) was applied. All the statistic calculations were performed with Statistica software version 6.0 (StatSoft Inc.).

2.5 Synchrotron based X-ray Absorption Spectroscopic measurements.

In order to obtain thin specimens for the μXRF measurements, wheat grains were immersed in 4 °C Milli-Q water. Then, the humected grains were embedded in paraffin and thin sections were cut using a microtome (MICROM HM 325 Rotary Microtome). The specimens were 60 μm thickness containing embryo, endosperm and outer layer.

μXRF mapping and μXANES measurements on the grain sections were performed at I18 beamline (Mosselmans et al., 2009) of Diamond Light Source using a 4-element Si drift fluorescence detector (Vortex). For the measurements, the specimens were mounted on top of carbon tape disk which was stuck on to a sapphire disk which was then glued onto the Al holder of the liquid Helium cryostat. The measurements were performed at 10 K to minimize the effects of the radiation damage. The spatial distribution of Se, Zn, Cu, Fe, K, Mn and Ca elements in the grain was obtained from the μXRF maps collected using an excitation energy of 12677 eV and a beam size of 20 μm. The step size used was 20 μm and the acquisition time per point was set to 0.05 s. The μXRF maps were processed using DAWN software (Basham et al., 2015). For shake of comparison, the maps were normalized to the maximum of counts on each grain for the element under study.

The tri-color maps were generated using the RGB mixer tool in DAWN which allows combining XRF maps of three different elements. The different intensity of the maps was balanced out to get the appropriated visualization of the three elements. μXANES spectra were collected at three different points of each part of the grain (embryo, endosperm and outer layer) to account for any possible inhomogeneities. The normalization of the μXANES spectra and the speciation analysis using linear combination fitting (LCF) was carried out with Athena program of the Demeter software package (Ravel and Newville, 2005) following standard procedures. For the LCF analysis, the XANES spectra of sodium selenite, sodium selenate, seleno-L-methionine, seleno-L-cystine and Se-(Methyl) selenocysteine hydrochloride (Sigma-Aldrich, Spain) measured in transmission mode were used as Se references since they are the species expected to be present in the plant. Further details about the measurements of the references and the LCF methodology followed can be found elsewhere (Xiao et al., 2020).

3. RESULTS AND DISCUSSION

3.1 Grain biomass

Biomass parameters, such as the average dry weight (DW) of single spikes (Figure 1a,b) and of grains per spike (Figure 1c,d), and the number of grains per spike (Figure 1e,f), were evaluated and compared among the different Se and biostimulant treatments to assess their effect on wheat development and yield.

Selenium treatments applied at the heading stage caused no significant effect on any of the biomass parameters studied except for Se(VI) that reduced significantly the number of grains produced per spike (Figure 1e). When Se was applied at the tillering stage, Se(VI) not only reduced the number of grains produced per spike but also the weight of both grains and spikes (Figure 1b,d,f).

Thus, Se(VI) is the Se species that caused the most negative influence on wheat yield specially when it was supplied during the production of tillers than at the later stage of heading. This is in agreement with the results found by Longchamp (Longchamp et al., 2015) who stated that the dry weight of *Zea mays* grains decreased by 60% and 80% in Se(VI)-dosed and Se(IV)-dosed plants, respectively,

compared to control grains. Oppositely, the results from Wang's (Wang et al., 2013) work support that Se(IV) could produce larger rice grains and higher yields.

At the heading stage, the application of the biostimulant (FA) clearly improved the biomass parameters under Se(VI) and Se(MIX) treatments to values significantly above NB ones (Figure 1a,c,e). Moreover, the biostimulant significantly increase the number of grains produced per spike under control conditions (No Se) as shown in Figure 1e. At tillering, the biostimulant counteracted the negative effects caused by Se(VI) on all the biomass parameters studied (Figure 1b,d,f), reaching similar values as the control treatment (NoSe, NB) and improving as well the weight of both spike and grain under the other Se treatments (Figure 1b,d). Although the nutrients are adequate during the plant growth, the extra Mo species from the biostimulant might enhance the mitochondria activity on the physiology of vegetal cells (Mendel and Kruse, 2012). It has also been pointed out that the biostimulant supplied in the nutrient solution may increase wheat biomass production due among other factors to the high level of Mo which is the essential for nitrogen acquisition and assimilation (Xiao et al., 2020). These results were expected since biostimulants are used to improve nutrient efficiency, abiotic stress tolerance and crop quality. Actually, the effect of biostimulants on plants' performance are often due to the combination and synergistic action of different compounds (Bulgari et al., 2015).

Wheat plants are more sensitive to Se in the form of Se(VI) when it is supplied at the tillering stage than when it is applied later on at the heading stage. This indicates that time of exposure (stage of application and length of treatment) to Se(VI) is an important factor to be considered because it diminishes the grain yield. In this context the biostimulant has a key role in reestablishing both the amount of grains produced per spike and their biomass (Figure 1b, d, f) as those obtained in control plants.

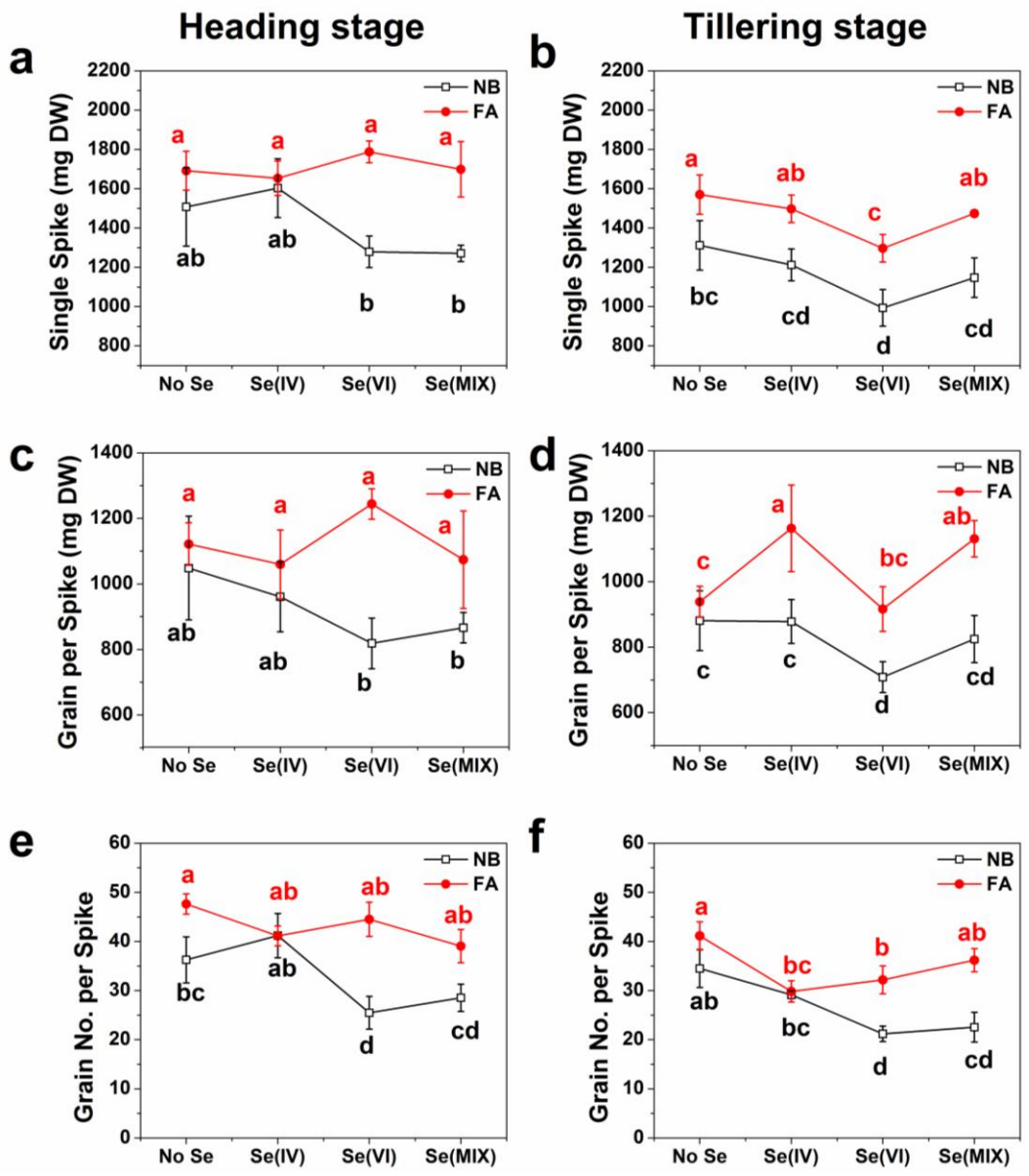


Figure 1. Grain biomass parameters of *T. aestivum* plants grown under different Se treatments (selenite, selenate and mixture of both selenium species (10 µM)) and biostimulant application (No biostimulant-NB, Foliar Application-FA) at different growth stages: Heading (a, c, e), Tillering (b, d, f). Results shown are means ± SE (n=4 plants). Different letters represent significant differences among groups (LSD). See text for details.

3.2 Total Selenium concentration in grain

The total Se levels found in grains for the different treatments indicate that Se-biofortification of grains was achieved with values within the range of 37-100 $\mu g \cdot g^{-1}$ DW and of 75-138 $\mu g \cdot g^{-1}$ DW for heading and tillering stages, respectively (Figure 2a, b).

The Se concentration in grains obtained from plants exposed to Se(IV) achieved similar levels (90-100 $\mu g \cdot g^{-1}$ DW) in both stages of application. In contrast, the total Se level in Se(VI) group was significantly higher in the tillering stage of application than in the heading stage, being these levels the highest of all the Se treatments, 125-138 $\mu g\ Se \cdot g^{-1}$ DW. Similarly, in the Se(MIX) group, due to the presence of Se(VI), total Se at tillering stage was found to be also higher, around 1.5-folds, than that of the heading stage. This is due to the fact that Se(IV) is rapidly assimilated into organic forms which are retained in roots, whereas Se(VI) is highly mobile in xylem transport and not readily converted into organic Se compounds (Cubadda et al., 2010; Curtin et al., 2006) and not only due to a longer exposure time determined by the stage of application.

Although the application of biostimulants is considered to promote Se accumulation in wheat grain (Peng et al., 2001), the increase observed in our study was only statistically significant for Se(VI) treatment at the heading stage of application (Figure 2a). Thus, the biostimulant does not increase Se accumulation in grains under the different Se treatments assayed but it influences other plant physiological parameters that enhances grain performance (weight and amount) counteracting the negative effects of an early Se exposure (tillering stage), especially in the form of Se(VI).

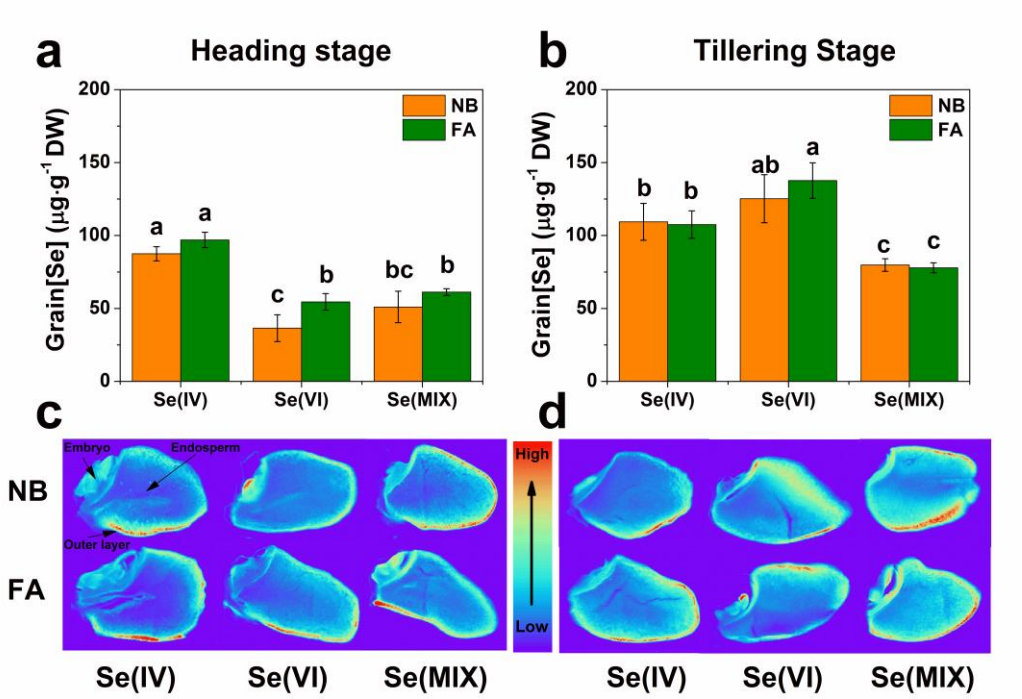


Figure 2. Total Se concentration (a, b) and X-ray fluorescence mapping of Se (c, d) in wheat grains grown under different treatments applied (No biostimulant-NB, Foliar Application-FA) at different growth stages: heading (a, c), tillering (b, d). The total concentration is displayed as mean±SE (n=3). Different letters represent significantly differences among groups (LSD). Warmer colors in XRF maps indicate higher Se concentration.

3.3 Selenium and nutrient distribution in grain by using µXRF mapping

Despite the valuable information extracted from the analysis of the total Se in the wheat grain, relevant information regarding the Se distribution in the grain is missing. In this regard, X-ray fluorescence (XRF) measurements using a micro-focused beam allow mapping grains sections providing a direct observation of the Se distribution in the different parts of the wheat grain (germ, endosperm and outer layer). As shown in the µXRF maps displayed in Figure 2c, d, Se is unevenly distributed in the grain (warmer colors indicate higher Se concentration). The higher concentrations of Se are mostly found in the germ and outer layer regardless the treatment applied. This is related to the fact that the outer layer, mostly the aleurone, and the germ are the main regions containing proteins and therefore Se-proteins assembled from seleno-aminoacids are located there (Gupta and Gupta, 2017; White, 2016). On the other hand, the images show much lower levels of Se

accumulation in the endosperm which is mostly constituted by starch and that contains a small fraction of fibers and proteins.

In addition, μXRF provides simultaneous information of the spatial distribution of several elements accumulated in the grain. In our study, the μXRF images for all the treatments show similar elemental distribution as the one displayed in Figure 3 for the Se(VI) applied at heading treatment (similar comparatives for the rest of the treatments can be found in Figure S2). The analysis of the μXRF maps indicates that aleurone and scutellum are major storage tissues for macro (P, K, Ca and Mg) as well as micro (Fe, Zn, Cu and Mn) nutrients (Singh et al., 2014). This distribution is quite consistent, and it does not get affected by neither Se species supplied in the treatment nor the application of plant biostimulants at different growth stage.

Tricolor RGB map helps to visualize the distribution patterns and co-localization of the nutrients and Se. As shown in Figure 3, K, Ca, Fe, Zn, Cu and Mn are located mostly in the embryo and the outer layer covering the aleurone, seed coat and pericarp (Singh et al., 2014). Selenium overlaps with them in some areas of the outer layer, but, from the tricolor image, we can distinguish that Se is mostly located in the most inner layer which it could be identified as the aleurone that is the part of the outer layer containing higher level of proteins (Brouns et al., 2012).

This knowledge of the grain tissue-specific element storage pattern can be useful in cereal processing to achieve a more efficient consumption of nutrients (Cserhalmi, 2002). Indeed, despite that the outer layer is a reservoir of minerals in wheat grain (Shewry, 2009), most of them are lost during the mechanical processing of wheat flour (Cakmak, 2008), which is not often consumed by people.

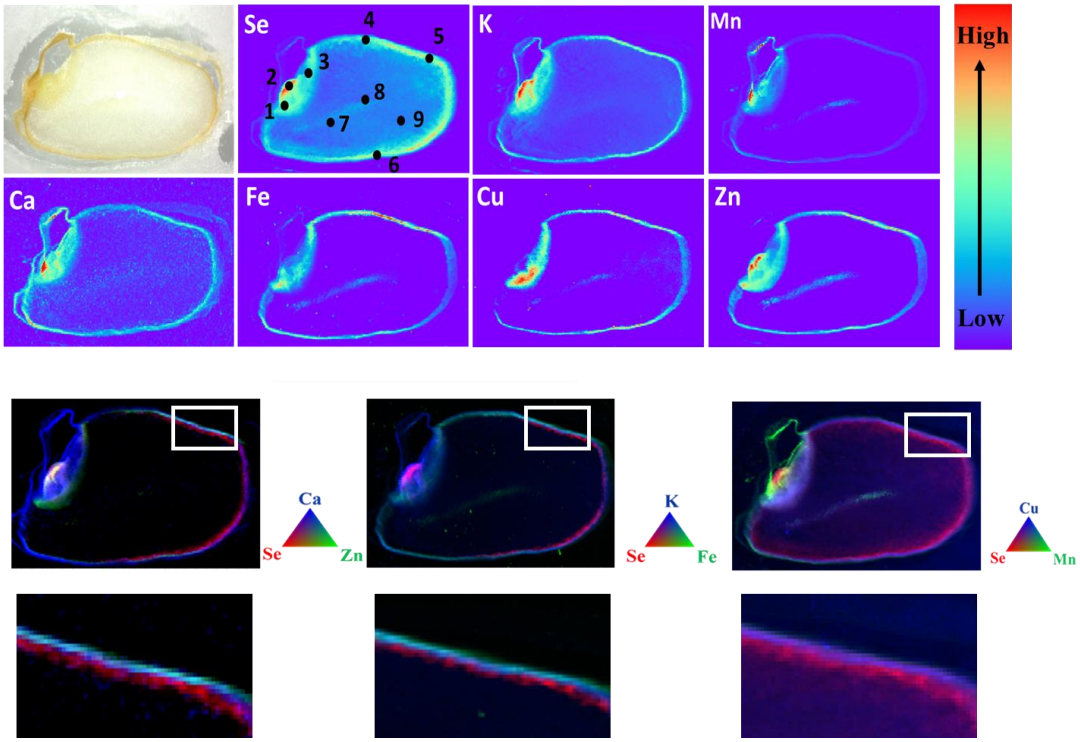
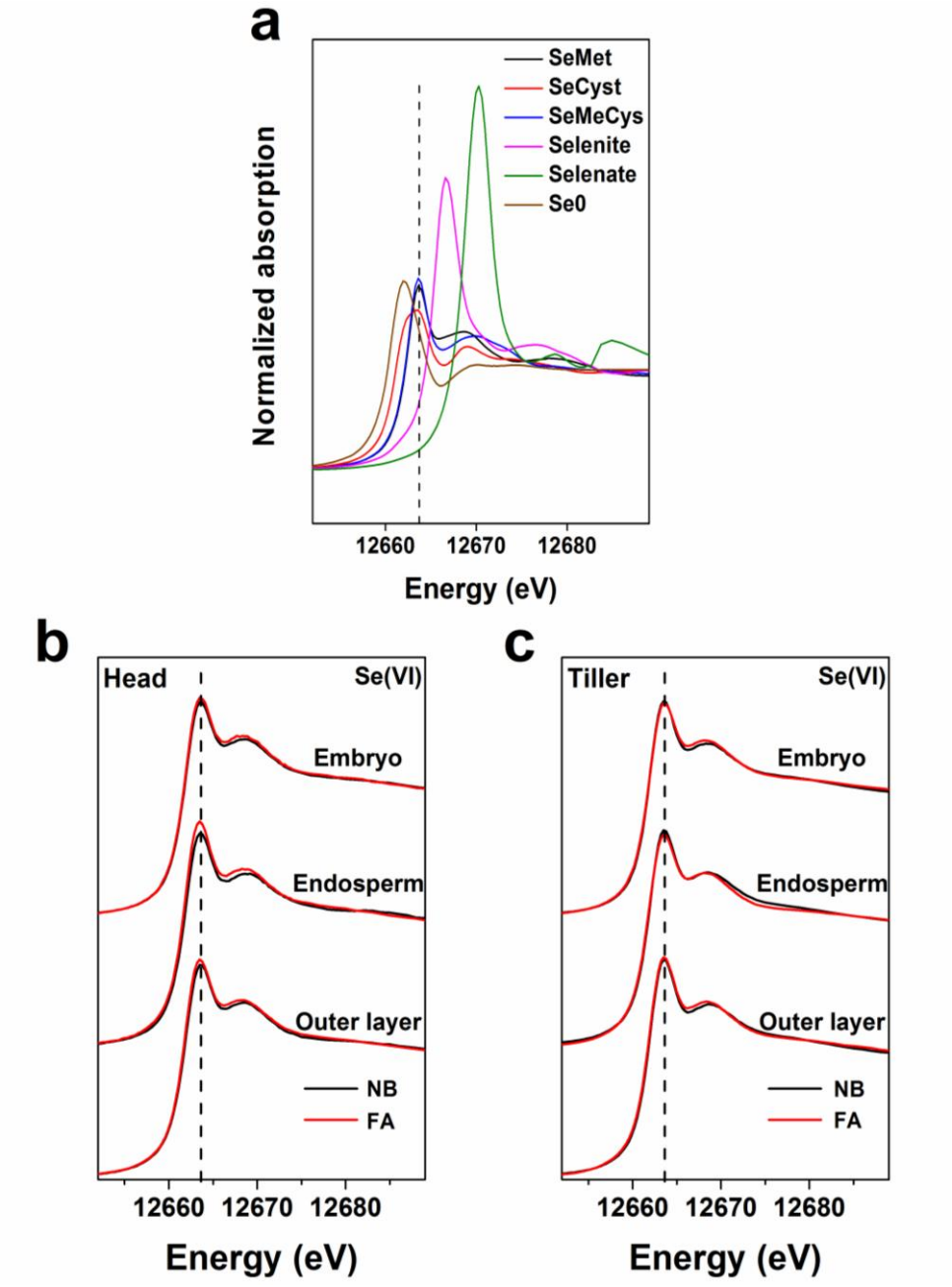


Figure 3. Normalized μXRF elemental maps of wheat grains for Se(VI) applied at heading stage. Warmer colors indicate higher element concentration. Top two rows: individual element distribution maps and optical microscope image (top left). Bottom two rows: tri-color merged images and corresponding enlarged areas. Colored triangle scales indicate the relative locations of elements color merged. The points marked in the Se μXRF image denote the positions where the μXANES were measured at the different parts of the grains (1-3 embryo, 4-6 outer layer, 7-8 endosperm).

3.4 Selenium speciation in grain determined by μXANES

The level of Se accumulation, its localization in tissues within the grain together with other nutrients, and ultimately the chemical form of Se determine its dietary availability in cereals (Singh et al., 2014). Hence, it is important to understand how Se speciation might be affected when Se is co-located with other elements present in the grain for the different treatments. In order to compare the Se speciation in the different grain tissues μXANES measurements were acquired at selected points of embryo, endosperm and outer layer. Figure 4b, c displays the comparative for all Se(VI) treatments as a representative case of study. The spectra collected on the grains were compared with Se references samples (Figure 4a): seleno-amino acids

(SeMet, SeCys, SeMeCys) and inorganic Se compounds (Se(0), Se(IV), Se(VI)). All the samples display a similar spectral profile characterized by a prominent white line at 12663.7 eV (marked with a vertical dashed line) which can be identified with compounds containing C-Se-C bond (e.g. SeMet or SeMeCys). The subtle spectral differences found among treatments suggest that the ratio among Se species may not change much. Indeed, the biostimulat application (FA) has some mild effect on the spectra respect NB in all the parts of the plant. On the other hand, little differences are observed when comparing the different parts of the grain (embryo, endosperm and outer layer) for the same treatment.



344

345 **Figure 4.** Normalized Se K-edge XANES spectra of Se references (a) and wheat
346 grain grown under Se(VI) and biostimulant treatments (No biostimulant-NB, Foliar
347 Application-FA) applied at different growth stage stages: heading (Head) (b), and
348 tillering (Tiller) (c). The spectra for embryo, endosperm and outer layer have been
349 shifted vertically for shake of comparison. Vertical line denotes to the white-line
350 position of species containing a C-Se-C bond (e.g. SeMet or SeMeCys).

351

352 Characterizing the ratio of the Se species contained in the wheat grain to get an
353 insight about the ratio of the seleno-amino acids formed is not only important to

354 understand Se mechanism in plant, but also essential to determine the benefits of
355 Se-enriched food for human health since different seleno-amino acids are
356 differently assimilated by the human body and they fulfill distinguished functions
357 related with specific health benefits. Indeed, to get a more quantitative information
358 of the Se species present in the grain, a linear combination fitting (LCF) analysis
359 has been performed using the afore mentioned Se references as standards, see
360 Figure 5. The values obtained from the LCF analysis have been included in Tables
361 S1 and S2 of the supporting information.
362
363 Figure 5a reports the ratio between inorganic and organic species for the NB
364 treatment applied at the heading and tillering stages. These results confirm that the
365 organic Se species are the main component in Se-biofortified wheat grains and that
366 FA treatment did not significantly influence this ratio (see Figure S3). These
367 observations are in agreement with previous studies reporting that the organic Se
368 species are the main Se species present in wheat grain (Eiche et al., 2015; Li et al.,
369 2008). This comparative also shows that the application of Se at different stages of
370 the plant growth affects the proportion of organic Se in wheat grains. The amount
371 of organic Se species found are always larger than 90% when the treatment is
372 applied at the tillering stage, whereas for the heading stage they are lower than 80%
373 in most of the cases. This indicates that the Se exposure stage and the length of the
374 treatment are important parameters in the conversion of inorganic Se to organic Se,
375 even in those cases reaching similar Se enrichment level (e.g. Se(IV) treatment).
376
377 A better insight in the composition is achieved when inspecting each independent
378 Se species included in the LCF analysis. As shown in **(**Figure 5b**)**, Se organic
379 species containing a C-Se-C bond (SeMet and SeMeCys) are the main compounds
380 distributed in the different parts of the grain when the Se treatment is applied at
381 heading stage. However, when Se is applied at tillering stage (Figure 5c) the amount
382 of C-Se-C species is lower than in the heading stage group. In addition, grains from
383 plants under biostimulant treatment (FA) seems to accumulate more C-Se-C amino
384 acids and elemental Se in comparison with the control group (NB) when Se is
385 applied at heading stage (Figure 5b), even though the total amount of organic
386 species remains very similar for both treatments. Hence, the amount of C-Se-Se-C
387 (SeCyst) amino acid in NB is slightly larger than in FA ones in heading stage group.

It has been pointed out that SeCyst found in the plant are usually due to the oxidation of SeCys since it is readily oxidized during the samples processing (Chan et al., 2010). Thus, the level of SeCyst found reflects the original level of SeCys in the plant.

Although both C-Se-C and SeCys species can be incorporated into proteins in place of methionine and cysteine, leading to toxicity, C-Se-C species have less harmful effects, since the incorporation of SeCys into the protein could interfere with the formation of disulfide bridge affecting tertiary structure of S-proteins (Terry et al., 2000). Our results show that when the Se treatment is applied at the heading stage, the Se toxicity is less severe than when applied at the tillering stage. The effect found in the grain is that the total Se content decreases together with the total organic Se, and there is an increase of C-Se-C respect to the total organic Se found in the heading group. Although FA group contains more C-Se-C and elemental Se than NB treatment in heading group, the contribution of FA in the Se tolerance is too mild to be conclusive.

By comparing Figure 5b and 5c, it can be noticed that Se(0) is only detected in the heading stage group of grains and it is negligible in the tillering ones. Se(0) is one of the product derived from SeCys via the action of a selenocysteine lyase (SL). Elemental Se is comparatively innocuous, therefore this could be a potential Se detoxification mechanism (Clemens, 2010; Van Hoewyk et al., 2005). This also supports the idea that when applying Se at the heading stage, the Se toxicity in wheat could be minimized due to the lower duration of the Se treatment (i.e., the number of applications are reduced) compared with the tillering stage application group. In the heading group, the abiotic stress caused by Se when the grain spike is just appearing may stimulate the expression of SL in order to enhance Se tolerance and maintain the growth cycle.

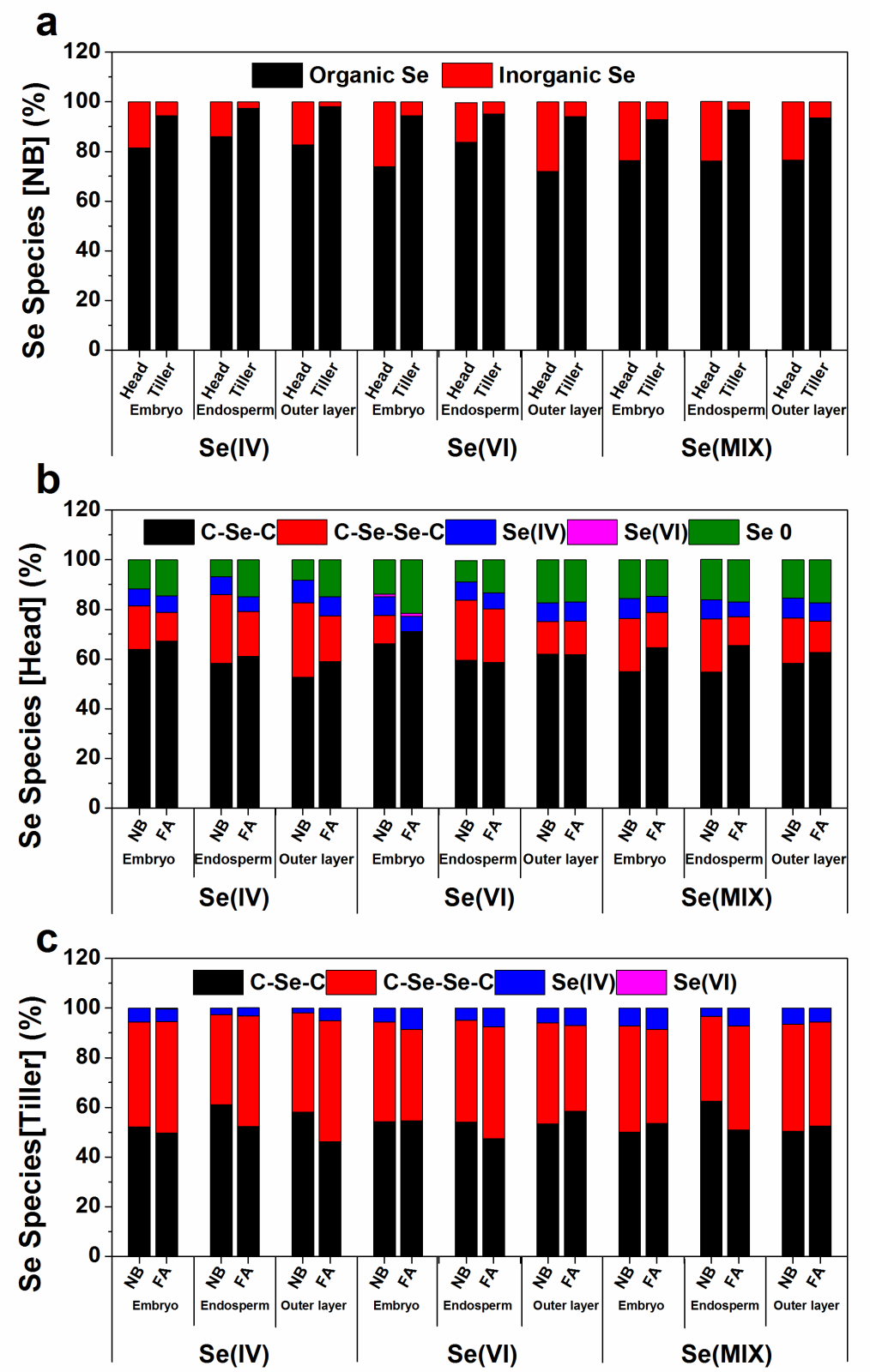


Figure. 5 Results from the linear combination fitting analysis of the μXANES spectra collected at different parts of the wheat grain: organic and inorganic Se species comparison, (a); Se species for heading, Head, (b); and tillering, Tiller, (c) application stages. See text for details.

4. CONCLUSIONS

Our results show that the biostimulant have a key role increasing both the amount of grains produced per spike and their biomass (DW) without diminishing the total amount of Se and/or disrupting Se species present in the grain, which is the main objective of biofortification processes. This is due to the combination and synergistic action of different compounds of biostimulant, it is also probably due to the catalytic influence of the Mo species from the biostimulant on the physiology of vegetal cells through the enhancement of the mitochondria activity.

While only when Se(VI) was supplied at the tillering stage, the highest Se levels present in the grain causes negative effects on wheat grain performance. Se-biofortification of the wheat grain was achieved in both in Se stage of application, heading and tillering, whereas when the Se treatment is applied at heading stage, it seems to minimize the Se induced toxicity regardless the Se species used. This is due to the lower duration of Se treatment compared to the tillering stage application group.

Our study shows that organic Se species are the main species found in wheat grain and that they are co-located with minerals in the outer layer and embryo parts of the grain which contain higher fraction of proteins. This distribution does not get affected by neither Se species supplied in the treatment nor the application of plant biostimulant at different growth stages. The amount of organic Se species are always larger than 90% when the treatment is applied at the tillering stage, whereas for heading stage they are lower than 80% in most of the cases. Grain from plant treated at the tillering application contains higher ratio of C-Se-C and lower C-Se-Se-C than grain treated at heading stage for which the ratio of C-Se-C and C-Se-Se-C is almost the same.

These results obtained from hydroponic cultivation set the basis for future studies on soil cultures since the valuable information obtained about how the Se toxicity influences the yield depending on the growing stage at which the Se is applied will be relevant for practical applications.

ABBREVIATIONS USED

FA, Biostimulant Foliar Application; Head, Heading stage; LCF, Linear Combination Fitting; MES, 2-Morpholinoethanesulphonic acid; NB, No Biostimulants; Se(IV), Sodium Selenite; Se(VI), Sodium Selenate; Se(MIX), 50%Sodium Selenite + 50%Sodium Selenate; SeMet, SelenoMehtionine; SeCyst, SelenoCystine; SeCys, SelenoCysteine; SeMeCys, Se-MethylSelenoCysteine; Tiller, Tillering stage; XRF, X-Ray Fluorescence; XAS, X-ray absorption spectroscopy.

DECLARATION OF COMPETING INTEREST

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

ACKNOWLEDGEMENTS

This research was supported by the Spanish CTM2015-65414-C2-1-R project from MINECO. We acknowledge Diamond light source beamtime no. SP18671-1 at I18 beamline. Roberto Boada acknowledges funding support from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 665919. Tingting Xiao acknowledges the grant from China Scholarship Council (201608330235). We thank Dr. Tina Geraki and Jorge Rodríguez-Martínez for her help during the μXRF experiments at I18 beamline (Diamond Light Source), and Rosa Padilla from the plant physiology group for her support.

AUTHOR CONTRIBUTIONS

T.X. contributed to the experimental design and setup, lab processing of samples, data analysis, manuscript writing and discussion. M.V and M.L contributed equally to the experimental design, data interpretation and in the writing and discussion of the manuscript. R.B. contributed to XANES and μXRF mapping data analysis and interpretation as well as manuscript writing and discussion. All authors read and approved the manuscript.

REFERENCES

Arnon, D.I., Hoagland, D.R., 1940. Crop production in artifical culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Science 50, 463–485.

Basham, M., Filik, J., Wharmby, M.T., Chang, P.C.Y., El Kassaby, B., Gerring, M., Aishima, J., Levik, K., Pulford, B.C.A., Sikharulidze, I., Sneddon, D., Webber, M., Dhesi, S.S., Maccherozzi, F., Svensson, O., Brockhauser, S., Náray, G., Ashton, A.W., 2015. Data Analysis WorkbeNch (DAWN). Journal of Synchrotron Radiation 22, 853–858. https://doi.org/10.1107/S1600577515002283.

Brouns, F., Hemery, Y., Price, R., Anson, N.M., 2012. Wheat Aleurone: Separation, Composition, Health Aspects, and Potential Food Use. Critical Reviews in Food Science and Nutrition 52, 553–568. https://doi.org/10.1080/10408398.2011.589540.

Buchanan, B.B., 2001. Genetic engineering and the allergy issue. Plant Physiology 126, 5–7. https://doi.org/10.1104/pp.126.1.5

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., Ferrante, A., 2015. Biostimulants and crop responses: A review. Biological Agriculture and Horticulture 31, 1–17. https://doi.org/10.1080/01448765.2014.964649

Cakmak, I., 2008. Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? Plant and Soil 302, 1–17. https://doi.org/10.1007/s11104-007-9466-3

Calvo, P., Nelson, L., Kloepper, J.W., 2014. Agricultural uses of plant biostimulants. Plant and Soil 383, 3–41. https://doi.org/10.1007/s11104-014-2131-8

Chan, Q., Afton, S.E., Caruso, J.A., 2010. Selenium speciation profiles in selenite-enriched soybean (*Glycine max*) by HPLC-ICPMS and ESI-ITMS. Metallomics 2, 147–153. https://doi.org/10.1039/b916194e

Chen, J., 2012. An original discovery: Selenium deficiency and Keshan disease (an endemic heart disease). Asia Pacific Journal of Clinical Nutrition 21, 320. https://doi.org/10.6133/apjcn.2012.21.3.01

Clemens, S., 2010. Cell Biology of Metals and Nutrients, Cell Biology of Metals and Nutrients. https://doi.org/10.1007/978-3-642-10613-2

Cserhalmi, Z., 2002. Cereals processing technology, Acta Alimentaria. https://doi.org/10.1556/aalim.31.2002.2.11

Cubadda, F., Aureli, F., Ciardullo, S., D'Amato, M., Raggi, A., Acharya, R., Reddy, R.A.V., Prakash, N.T., 2010. Changes in selenium speciation associated with increasing tissue concentrations of selenium in wheat grain. Journal of Agricultural and Food Chemistry 58, 2295–2301. https://doi.org/10.1021/jf903004a

Curtin, D., Hanson, R., Lindley, T.N., Butler, R.C., 2006. Selenium concentration in wheat (*Triticum aestivum*) grain as influenced by method, rate, and timing of sodium selenate application. New Zealand Journal of Crop and Horticultural Science 34, 329–339. https://doi.org/10.1080/01140671.2006.9514423

Daniels, L.A., 1996. Selenium metabolism and bioavailability. Biological Trace Element Research 54, 185–199. https://doi.org/10.1007/BF02784430

Eiche, E., Bardelli, F., Nothstein, A.K., Charlet, L., Göttlicher, J., Steininger, R., Dhillon, K.S., Sadana, U.S., 2015. Selenium distribution and speciation in plant parts of wheat (*Triticum aestivum*) and Indian mustard (*Brassica juncea*) from a seleniferous area of Punjab, India. Science of the Total Environment 505, 952–961. https://doi.org/10.1016/j.scitotenv.2014.10.080

Ellis, D.R., Salt, D.E., 2003. Plants, selenium and human health. Current Opinion in Plant Biology 6, 273–279. https://doi.org/10.1016/S1369-5266(03)00030-X

Fordyce, F.M., 2013. Selenium deficiency and toxicity in the environment, in: Essentials of Medical Geology: Revised Edition. Springer, London, pp. 375–416. https://doi.org/10.1007/978-94-007-4375-5_16

Guerrero, B., Llugany, M., Palacios, O., Valiente, M., 2014. Dual effects of different selenium species on wheat. Plant Physiology and Biochemistry 83, 300–307. https://doi.org/10.1016/j.plaphy.2014.08.009

Gupta, M., Gupta, S., 2017. An Overview of Selenium Uptake, Metabolism, and Toxicity in Plants. Frontiers in Plant Science 7, 2074. https://doi.org/10.3389/fpls.2016.02074

James, L.F., Panter, K.E., Mayland, H.F., Miller, M.R., Baker, D.C., 1989. Selenium Poisoning in Livestock: A Review and Progress, in: Selenium in Agriculture and the Environment Seleniuminagric. Soil Science Society of America and American Society of Agronomy, Madison, WI, pp. 123–131. https://doi.org/10.2136/sssaspecpub23.c7

Li, H.F., McGrath, S.P., Zhao, F.J., 2008. Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytologist 178, 92–102. https://doi.org/10.1111/j.1469-8137.2007.02343.x

Longchamp, M., Castrec-Rouelle, M., Biron, P., Bariac, T., 2015. Variations in the accumulation, localization and rate of metabolization of selenium in mature *Zea mays* plants supplied with selenite or selenate. Food Chemistry. https://doi.org/10.1016/j.foodchem.2015.02.137

Lüttge, U., 1962. Progress in botany, Nature. https://doi.org/10.1038/1941023a0

Mendel, R.R., Kruse, T., 2012. Cell biology of molybdenum in plants and humans. Biochimica et Biophysica Acta - Molecular Cell Research 1823, 1568–1579. https://doi.org/10.1016/j.bbamcr.2012.02.007

Mosselmans, J.F.W., Quinn, P.D., Dent, A.J., Cavill, S.A., Moreno, S.D., Peach, A., Leicester, P.J., Keylock, S.J., Gregory, S.R., Atkinson, K.D., Rosell, J.R., 2009. I18 - The microfocus spectroscopy beamline at the Diamond Light Source. Journal of Synchrotron Radiation 16, 818–824. https://doi.org/10.1107/S0909049509032282

Navarro-Alarcon, M., Cabrera-Vique, C., 2008. Selenium in food and the human body: A review. Science of the Total Environment 400, 115–141. https://doi.org/10.1016/j.scitotenv.2008.06.024

Peng, A., Xu, Y., Wang, Z.J., 2001. The effect of fulvic acid on the dose effect of selenite on the growth of wheat. Biological Trace Element Research 83, 275–279.

https://doi.org/10.1385/BTER:83:3:275
Ravel, B., Newville, M., 2005. ATHENA, ARTEMIS, HEPHAESTUS: Data analysis for X-ray absorption spectroscopy using IFEFFIT. Journal of Synchrotron Radiation 12, 537–541. https://doi.org/10.1107/S0909049505012719
Rayman, M.P., 2000. The importance of selenium to human health. Lancet 356, 233–241. https://doi.org/10.1016/S0140-6736(00)02490-9
Shewry, P.R., 2009. Wheat. Journal of Experimental Botany 60, 1537–1553. https://doi.org/10.1093/jxb/erp058
Singh, S.P., Vogel-Mikuš, K., Vavpetič, P., Jeromel, L., Pelicon, P., Kumar, J., Tuli, R., 2014. Spatial X-ray fluorescence micro-imaging of minerals in grain tissues of wheat and related genotypes. Planta 240, 277–289. https://doi.org/10.1007/s00425-014-2084-4
Terry, N., Zayed, A.M., de Souza, M.P., Tarun, A.S., 2000. Selenium in Higher Plants. Annual Review. Plant Physiol. Plant Mol. Biol. 51, 401–432. https://doi.org/10.1080/00378941.1870.10829847
Thomson, C.D., 1998. Selenium speciation in human body fluids. Analyst 123, 827–831. https://doi.org/10.1039/a707292i
Toulis, K.A., Anastasilakis, A.D., Tzellos, T.G., Goulis, D.G., Kouvelas, D., 2010. Selenium supplementation in the treatment of Hashimoto's thyroiditis: A systematic review and a meta-analysis. Thyroid 26, 1681–1692. https://doi.org/10.1089/thy.2009.0351
Van Hoewyk, D., Garifullina, G.F., Ackley, A.R., Abdel-Ghany, S.E., Marcus, M.A., Fakra, S., Ishiyama, K., Inoue, E., Pilon, M., Takahashi, H., Pilon-Smits, E.A.H., 2005. Overexpression of AtCpNifS enhances selenium tolerance and accumulation in arabidopsis. Plant Physiology 139, 1518–1538. https://doi.org/10.1104/pp.105.068684
Varo, P., Alfthan, G., Ekholm, P., Aro, A., Koivistoinen, P., 1988. Selenium intake and serum seleniun in Finland: Effects of soil fertilization with selenium. American Journal of Clinical Nutrition 48, 324–329. https://doi.org/10.1093/ajcn/48.2.324
Wang, Y.D., Wang, X., Wong, Y.S., 2013. Generation of selenium-enriched rice with enhanced grain yield, selenium content and bioavailability through fertilisation with selenite. Food Chemistry 141, 2385–2393. https://doi.org/10.1016/j.foodchem.2013.05.095
Weekley, C.M., Aitken, J.B., Musgrave, I.F., Harris, H.H., 2012. Methylselenocysteine treatment leads to diselenide formation in human cancer cells: Evidence from X-ray absorption spectroscopy studies. Biochemistry 51, 736–738. https://doi.org/10.1021/bi201462u
White, P.J., 2016. Selenium accumulation by plants. Annals of Botany 117, 217–235. https://doi.org/10.1093/aob/mcv180
Xiao, T., Boada, R., Marini, C., Llugany, M., Valiente, M., 2020. Influence of a plant biostimulant on the uptake, distribution and speciation of Se in Se-enriched wheat (*Triticum aestivum* L. cv. Pinzón). Plant and Soil 455, 409–423. https://doi.org/10.1007/s11104-020-04686-y