Universitat Autònoma de Barcelona

Chemical and synchrotron techniques for the characterization and development of functional foods. Plant biostimulant effects on Se enriching wheat

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Summary

Selenium (Se) is vital to human health as it is required for a variety of biochemical and physiological processes in the human body, such as antioxidant defenses, immune function and formation of sperm. The bioavailability of Se is intimately related with its chemical form. In general, organic Se species are more bioavailable than inorganic ones, for which the bioavailability can reach values up to 80-90%, whereas only 20% of inorganic Se is able to reach to the circulatory system in human body. The resources of Se supplement from daily food are diverse, largely obtained from edible plants since the human body cannot synthesize assimilable organic Se species effectively. Selenite (Se(IV)) and selenate (Se(VI)) are the predominant inorganic Se forms that are taken up by plants. Afterwards, selenite (Se(IV)) and selenate (Se(VI)) are mostly metabolized to organic forms which are the forms of Se effectively assimilable for animals and human intake. Although crop enrichment with Se-containing fertilizers to obtain Se-biofortified food in Se-deficient regions is becoming an increasingly common practice, there are still issues to be addressed regarding the observed Se-induced toxicity to the plant itself. In this respect, plant biostimulants are used to enhance nutrition efficiency, abiotic stress tolerance and crop quality. In this study, in order to overcome the Se-induced stress on wheat plants grown hydroponically, we have applied a biostimulant, based on a complex of hybrid hetero-polyoxometalates of Keggin structure molecules mixed with humic acids, called “Phyto-Fitness” (BIO Fitos S.R.O., Czech Republic). Our aim is to assess the effect of this product in counteracting the toxicity of Se which hampers the normal development of wheat plants, and second, to evaluate a possible modification on the Se speciation in the presence of the biostimulant.

Xylem sap analysis showed that inorganic Se speciation, Se(IV) and Se(VI), has different metabolic pathway in plant tissue. Translocation from root to shoot is a key process to control Se and nutrient elements accumulation in wheat aerial part. Furthermore, the studies regarding the application of the biostimulant on wheat plants are performed on short-term and long-term plant growth periods.

Xylem sap samples were collected to investigate if selenium species affect pH and the translocation of mineral elements from root to shoot. In this concern, the levels of the micronutrients Zn, Cu and Mn decreased in the xylem sap in the presence of Se(IV) species, consequently, their accumulation in shoot was similar to that of the xylem sap. A quick and low-cost method based on acid-base potentiometric titrations for testing the xylem sap response to the Se species has been established.

The study of Se enrichment in short-term wheat plants was carried out with plants grown hydroponically and exposed to either Se(IV), Se(VI) or a mixture of both species (Se(MIX)) in presence or absence of the biostimulant by either foliar, FA; or root application, RA, during 2 weeks. Biomass, mineral nutrient concentration, phytohormones and Se speciation were investigated. Our results show that the biostimulant FA did not modify the plant biomass but RA significantly increased the root biomass in all treatments as well as the shoot biomass of plants exposed to Se(VI) and Se(MIX) even when both biostimulant modes of application caused a severe reduction of indole acetic acid (IAA) levels in shoots. The biostimulant accelerated the translocation of Se from roots to shoots in the presence of Se(VI) and Se(MIX), and only had a noticeable influence on the Se speciation in roots, not much in shoots. X-ray absorption spectroscopy allowed to identify organic Se as the main Se species formed in the plant shoots being the influence of the plant biostimulant almost negligible on the Se speciation.

These results indicate the potential of this biostimulant in the Se-enrichment of crops while avoiding the possible stress induced by this element.

Long-term wheat plants were grown under the same environmental conditions and Se treatments as the short-term ones, but the investigations were targeted on the co-application of the Se-treatments and the biostimulant foliar application (FA) at different growth stages (tillering stage or heading stage) until harvesting. We determined total Se content in grain, grain biomass and we also used micro focus X-ray spectroscopy techniques (μ-XRF mapping and μ-XAS) to get a better insight in the distribution of minerals such as Fe, Ca, Mn, K etc. and of the speciation of Se in different regions of wheat grain. Our study proves that the biostimulant had a key role in enhancement of both the amount of grains produced per spike and their biomass (DW) without diminishing the total amount of Se and maintaining Se speciation as in absence of biostimulant. The use of micro focused X-ray spectroscopy techniques allows us to understand that organic Se is the main Se species in wheat grain and that, Se application stage influence the proportion of organic Se. This information will be useful to minimize both plant toxicity and economic costs towards a more effective Se supplementation.

The fact of overcoming or diminishing the toxicity induced by Se application has a great importance in agriculture and in biofortification programs since the productivity of crops would not be affected and the necessary dietary amount of organic Se forms in edible parts will reach an adequate range.

Resumen

El selenio (Se) es vital para la salud humana, ya que se requiere para una variedad de procesos bioquímicos y fisiológicos en el cuerpo humano, tales como las defensas antioxidantes, la función inmunológica y la formación de esperma. La biodisponibilidad del Se está íntimamente relacionada con su forma química. En general, las especies de Se orgánicas están más biodisponibles que las inorgánicas, por lo que la biodisponibilidad puede alcanzar valores de hasta el 80-90%, mientras que sólo el 20% del Se inorgánico es capaz de llegar al sistema circulatorio del cuerpo humano. Las fuentes de suplemento de Se en la alimentación diaria son varias, y se obtienen en gran medida del consumo de vegetales, ya que el cuerpo humano no puede sintetizar eficazmente las especies de Se orgánico asimilables. El selenito (Se(IV)) y el selenato (Se(VI)) son las formas inorgánicas predominantes de Se que las plantas pueden absorber y metabolizar en su mayor parte en formas orgánicas que son las formas de Se eficientemente asimilables para los animales y la ingesta humana. El uso de fertilizantes que contienen Se se está convirtiendo en una práctica cada vez más común en regiones con suelos deficientes en este elemento para poder obtener cultivos y alimentos enriquecidos con Se. Pese a ello, todavía hay cuestiones que deben abordarse en relación con la toxicidad inducida por el Se en la propia planta. A este respecto, los bioestimulantes vegetales se utilizan para mejorar la eficiencia de la nutrición, la tolerancia al estrés abiótico y la calidad de los cultivos. En este estudio, para superar el estrés inducido por el Se en las plantas de trigo cultivadas hidropónicamente, hemos aplicado un bioestimulante, basado en un complejo de heteropolioxometalatos híbridos de moléculas de la estructura de Keggin mezcladas con ácidos húmicos, llamado "Phyto-Fitness" (BIO Fitos S.R.O., República Checa). Nuestro objetivo es evaluar el efecto de este producto para contrarrestar la toxicidad del

Se que impide el desarrollo normal de las plantas de trigo y, en segundo lugar, evaluar una posible modificación de la especiación del Se en presencia del bioestimulante.

El estudio de la savia del xilema demostró que la especiación inorgánica de Se, Se(IV) y Se(VI) tiene una vía metabólica diferente en el tejido vegetal. La translocación de la raíz al tallo es un proceso clave para controlar la acumulación de Se y de elementos nutritivos en la parte aérea del trigo. Además, los estudios relativos a la aplicación del bioestimulante en las plantas de trigo se realizaron en distintos períodos de crecimiento de las plantas a corto y a largo plazo.

Se recogieron muestras de savia del xilema para investigar si las especies de Se afectan al pH y a la translocación de elementos minerales de la raíz al tallo. En este aspecto, los micronutrientes Zn, Cu y Mn en la savia del xilema disminuyeron en presencia de Se(IV), por lo que el patrón de acumulación de elementos en el tallo fue similar al de la concentración de elementos en la savia del xilema. Se ha establecido un método rápido y de bajo costo basado en titriaciones potenciométricas ácido-base para ensayar la respuesta de la savia del xilema a la especie Se.

El ensayo del enriquecimiento de Se en plantas de trigo a corto plazo se llevó a cabo en plantas cultivadas hidropónicamente y expuestas a Se(IV), Se(VI) o a una mezcla de ambas especies (Se(MIX)) tanto en presencia como en ausencia del bioestimulante, aplicado por vía foliar (FA) o por vía radicular (RA) durante 2 semanas. Se investigaron la biomasa, la concentración de nutrientes minerales, las fitohormonas y la especiación de Se. Nuestros resultados muestran que FA no modificó la biomasa de la planta, mientras que RA aumentó significativamente la biomasa de las raíces en todos los tratamientos, así como la biomasa de los tallos de las plantas expuestas a Se(VI) y Se(MIX), incluso cuando ambos modos de aplicación del bioestimulante causaron una severa reducción de los niveles de ácido indol acètico (IAA) en los tallos. El bioestimulante aceleró la

translocación de Se de las raíces a los tallos en presencia de Se(VI) y Se(MIX), y sólo tuvo una influencia notable en la especiación de Se en las raíces, menor en los tallos. La espectroscopia de absorción de rayos X permitió identificar el Se orgánico como la principal especie de Se formada en los tallos, siendo la influencia del bioestimulante casi insignificante en la especiación de Se. Estos resultados indican el potencial de este biostimulant en el enriquecimiento con Se de los cultivos evitando al mismo tiempo el posible estrés inducido por este elemento.

Las plantas de trigo para el ensayo a largo plazo se cultivaron bajo las mismas condiciones ambientales y los mismos tratamientos de Se que las de corto plazo, pero las investigaciones se centraron en la co-aplicación de los tratamientos de Se y de la aplicación foliar del bioestimulante (FA) en diferentes etapas de crecimiento del trigo (etapa de ahijamiento o etapa de emergencia de la inflorescencia) hasta la cosecha. Determinamos el contenido total de Se en el grano, la biomasa del grano y también utilizamos técnicas de espectroscopia de rayos X de microfocalización (μ-XRF mapping y μ-XAS) para obtener una mejor comprensión de la distribución de minerales como Fe, Ca, Mn, K etc., y la especiación de Se en diferentes regiones del grano de trigo. Nuestro estudio demuestra que el bioestimulante tuvo un papel clave en el aumento tanto de la cantidad de granos producidos por espiga como de su biomasa (DW) sin disminuir la cantidad total de Se y manteniendo la especiación de Se igual como en ausencia de bioestimulante. El uso de técnicas de espectroscopia de rayos X micro focalizada permite comprender que el Se orgánico es la principal especie de Se en el grano de trigo y que la etapa de aplicación del Se afecta a la proporción de este Se orgánico. Esta información será útil para minimizar tanto la toxicidad de la planta como los costos económicos para lograr una suplementación de Se más efectiva.

El hecho de superar o disminuir la toxicidad inducida por la aplicación de Se tiene una gran importancia en la agricultura y en los programas de biofortificación ya que la productividad de los cultivos no se vería afectada y la cantidad dietética necesaria de formas orgánicas de Se en las partes comestibles alcanzará un rango adecuado.

Resum

El seleni és essencial per a la salut humana, ja que es imprescindible en diversos processos bioquímics i fisiològics en el cos humà, com les defenses antioxidants, la funció immunològica i la formació de l'esperma. La biodisponibilitat del Se està íntimament relacionada amb la seva forma química. En general, les espècies de Se orgàniques són més biodisponibles que les inorgàniques, per la qual cosa la biodisponibilitat pot assolir valors de fins al 80-90%, mentre que només el 20% del Se inorgànic pot arribar al sistema circulatori del cos humà. Les fonts de suplement de Se en els aliments diaris són diverses, i s'obtenen en gran mesura del consum de vegetals, ja que el cos humà no pot sintetitzar de forma eficaç les espècies de Se orgànic assimilables. El selenit (Se (IV)) i el selenat (Se (VI)) són les formes inorgàniques predominants de Se que les plantes poden aborbir i metabolitzar en la seva major part cap a formes orgàniques que són les formes de Se eficientment assimilables per als animals i la ingesta humana. L'ús de fertilitzants que contenen Se s'està convertint en una pràctica cada vegada més comú en regions amb sòls deficients en aquest element per poder obtenir així cultius i aliments enriquits amb Se. Malgrat això, encara hi ha qüestions que s'han d'abordar en relació amb la toxicitat induïda pel Se en la pròpia planta. En aquest sentit, els bioestimulants vegetals s'utilitzen per millorar l'eficiència de la nutrició, la tolerància a l'estrès abiòtic i la qualitat dels cultius. En aquest estudi, per superar l'estrès induït pel Se en les plantes de blat conreades hidropònicament, hem aplicat un bioestimulant, basat en un conjunt d'heteropolioxometalats híbrids de molècules amb l'estructura de Keggin barrejades amb àcids húmics, anomenat "Phyto-Fitness" (BIO Fitos SRO, República Txeca). El nostre primer objectiu és avaluar l'efecte d'aquest producte per a contrarestar la toxicitat del Se que impedeix el correcte desenvolupament de les plantes i, en segon lloc, avaluar la possible modificació de l'especiació del Se degut a la presència del bioestimulant. La

investigació de la saba del xilema va demostrar que l'especiació inorgànica de Se, Se (IV) i Se (VI) té diferents vies metabòliques en el teixit vegetal. La translocació des de l'arrel cap a la tija és un procés clau per controlar l'acumulació d'elements nutritius i de Se a la part aèria del blat. A més, l'estudi de l'aplicació del biostimulant a les plantes de blat es va realitzar en diferents períodes de creixement de les plantes; a curt i llarg termini.

Es van recollir mostres de saba del xilema per investigar si les espècies de seleni afecten el pH i la translocació i acumulació de diversos elements minerals. En aquest aspecte, els micronutrients Zn, Cu i Mn van disminuir en presència de les espècies Se (IV), per tant, el patró d'acumulació d'elements en tija va ser similar al de la concentració d'elements trobats a la saba del xilema. S'ha establert un mètode ràpid i de baix cost, basat en titracions potenciomètriques d'àcid-base, per determinar la resposta de la saba del xilema a les espècies de Se.

L'estudi de l'enriquiment de Se en plantes de blat a curt termini es va realitzar en plantes cultivades hidropònicament i exposades a Se(IV), Se(VI) o a una barreja d'ambdues espècies (Se(MIX)) tant en presència com en absència del biostimulant, aplicat via foliar (FA) o a través de les arrels (RA), durant dues setmanes. Es va determinar la biomassa, la concentració de nutrients minerals, els nivells de fitohormones i l'especiació de Se. Els nostres resultats mostren que FA no va modificar la biomassa de la planta però que RA va fer augmentar significativament la biomassa de l'arrel en tots els tractaments, així com la biomassa de la tija de plantes exposades a Se(VI) i Se(MIX), fins i tot quan les dues vies d'aplicació del biostimulant van provocar una severa reducció dels nivells d'àcid indol acètic (IAA) a la part aèria. El biosestimulant va accelerar la translocació de Se des de les arrels cap a la part aèria en presència de Se(VI) i Se(MIX) i només va tenir una influència notable en l'especiació de Se en arrels, i poca en tija. L'espectroscòpia d'absorció de raigs X va permetre identificar el Se orgànic com a l'espècie principal de

Se formada a la part aèria, essent la influència del biostimulant gairebé insignificant en l'especiació Se. Aquests resultats indiquen el potencial d'aquest biostimulant en l'enriquiment amb Se dels conreus evitant al mateix temps el possible estrès induït per aquest element.

Les plantes de blat per l'assaig a llarg termini es van cultivar en les mateixes condicions ambientals i els mateixos tractaments de Se que les de curt termini, però les investigacions s'orienten a la co-aplicació dels tractaments de Se i l'aplicació foliar del biostimulant (FA) en diferents etapes de creixement (afillament i emergència de la inflorescència) fins a la collita. En aquest assaig es va determinar el contingut total de Se en gra, la biomassa de gra i també es van utilitzar tècniques d'espectroscòpia de raigs X microfocalitzada (mapatge μ-XRF i μ-XAS) per obtenir una millor visió de la distribució d'elements minerals com Fe, Ca, Mn, K, etc, i de l'especiació de Se en diferents regions del gra de blat. El nostre estudi demostra que el biostimulant va tenir un paper fonamental en l'increment tant de la quantitat de gra produït per espiga com de la seva biomassa (DW) sense disminuir la quantitat total de Se i mantenint la seva especiació igual que en absència del biostimulant. L'ús de tècniques d'espectroscòpia de raigs X microfocalitzada ens permet entendre que el Se orgànic és l'espècie principal de Se present en el gra de blat i que l'estadi de creixement en el que es realitza l'aplicació del Se afecta a la proporció d'aquest Se orgànic. Aquesta informació serà útil per minimitzar la toxicitat de les plantes i els costos econòmics per aconseguir un enriquiment de Se més eficient.

El fet de superar o disminuir la toxicitat induïda per l'aplicació Se té una gran importància en l'agricultura i en els programes de biofortificació ja que la productivitat dels cultius no es veuria afectada i la quantitat dietètica necessària de formes orgàniques de Se en parts comestibles assolirà un rang adequat.

Index

Abbreviations

CIM: Constant ionic media

DMSe: Dimethylselenide

DMDSe: Dimethyldiselenide

FA: Biostimulant Foliar Application

HPAs: Hybrid hetero-polyoxometalates

IAA: 3-Indoleacetic Acid (IAA)

IBA: Indole-3-Butyric Acid

IPyA: Indole-3-pyruvic acid;

LCF: Linear Combination Fitting

MES: 2-Morpholinoethanesulphonic acid

NB: No Biostimulants

RA: Biostimulant Root Application

ROS: Reactive Oxygen Species

SOD: Superoxide Dismutase

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

TMSe+: Trimethylselenonium

XRF: X-Ray Fluorescence

XAS: X-ray absorption spectroscopy

Chapter Ⅰ

Introduction

Introduction

1.1 Selenium

Se properties and oxidation states

Selenium (Se), with the symbol Se and the atomic number 34, belonging to chalcogen group of elements, has a strong physic-chemical resemblance with sulphur (S) [1].

Selenium has seven naturally isotopes, ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, which are stable. ^{80}Se being the most abundant (49.6% natural abundance) [2].

The most common oxidation states (OS) of selenium are -2, 0, +4 and +6. Selenium in -2 OS is found in organic Se, such as selenoaminoacids. Se 0 OS exists in elemental selenium, Se^{+4} OS exists in selenite salts such as Na_2SeO_3, and finally, Se^{+6} is found in selenate salts such as Na_2SeO_4.

Some historical data on selenium

Selenium (Greek *selene* meaning "Moon") was discovered in 1817 by Jöns Jacob Berzelius and Johan Gottlieb Gahn. They found that pyrite from the Falun mine created red precipitates in the lead chambers which gave off smell like horseradish when burned. Berzelius analyzed these precipitates that were similar to sulfur and to tellurium, and named the new element as the Moon (*selene*), following the same idea as with tellurium which was named for Earth (*Tellus*) [3].

In the mid-1870s, Se had been using as the industry commercial product by Werner Siemens, such as Se cell and dye of glass. However, typical diseases appeared when livestock animals in American West and plains states took plants containing high amounts of selenium. In 1935, Franle and Painter had investigated that Se had a compound very

similar to cystine [4], which was incorporated into protein resulting in toxicity to plants and animals. Therefore, Se was classified as toxin for a long time until it was discovered to be necessary for the activity of some bacteria [5] and essential to animals [6]. Karl schwarz [7] had found that Se acted as "factor 3" working with methionine and vitamin E for preventing the liver necrosis in rat.

1.2 Selenium and health

Selenium- "essential poison"

Selenium is an element that has a very small margin between demand levels and toxicity [8]. The US Department of agriculture has a R.D.A of 55 μg/day for adults [9], while the world health organization has established a toxic limit of 800μg/day for adults [10]. The recommendations for selenium intake are average 60 μg/day for men and 53 μg/day for woman [11].

The disease of selenium deficiency

Selenium deficiency occurs when the body does not have enough selenium. It is often happened in those regions that there are low amounts of Se in soil, such as Northeast China and Russia. Keshan disease is a typical and first noted symptom on the case of Se deficiency, which has discovered in Keshan county of Heilongjiang province, Northeast China in 1935 [12]. The patients of Keshan disease have myocardial necrosis, leading to weakening of the heart. Apart from Keshan disease, hypothyroidism, extreme fatigue, mental slowing, miscarriages et., are also diseases caused by Se deficiency [13].

Selenium essentiality: benefit for humans

Investigators believe that maintain an adequate Se level in human body would be beneficious for human health. Proper selenium intake can be antiviral, reduce the risk of autoimmune thyroid disease and cancer, is essential for successful male and female reproduction and increase immune system function.

Selenoproteins

Selenium in the human body has been found in the form of selenoaminoacids, which are aminoacids that have Se instead of S atoms in their chemical structure. The active form of Se in the human body are selenoproteins, where 25 of them have selenocysteine (SeCys) at their active center. The insertion of SeCys to form a selenoprotein is specified by UGA codon [14].

Table I-1. Selection of selenoproteins with known functions relevant to health. Data from [15–19].

Selenium protein	Function
Glutathione peroxidases (GPxs) GPX1 (cytosolic) GPX2 (gastrointestinal) GPX3 (plasma) GPX4 (phospholipid)	Antioxidant enzymes, remove hydrogen peroxide, lipid and phospholipid hydroperoxides, maintain the cell membrane integrity. Against cancer and enhance the male fertility.
Iodothyronine deiodinases	Catalyze the 5'5-mono-deiodination of the prohormone thyroxine(T4) to the active thyroid hormone 3,3'5-triodothyronine(T3).
Thioredoxin reductases	Prevention of some forms of cancer; Play a regulatory role in the metabolic activity of catalyzes the NADPH dependent reduction of thioredoxin.
Sperm mitochondrial capsule selenoprotein	Stability the integrity of the sperm flagella.
Selenoprotein H	Essential for viability and antioxidant defense.
Selenoprotein K	Participate in antioxidant defense, calcium regulation and in the endoplasmic reticulum associated protein degradation (ERAD) pathway.
Selenoprotein M	Protect against oxidative damage in the brain and may potentially function in calcium regulation.
Selenoprotein P	An important Se transporter Se; Be associated with cell membranes. Serve as an antioxidant.
Selenoprotein R	Thiol disulfide oxidoreductase that participates in the formation of disulfide bonds and can be implicated in calcium responses.
Selenoprotein S	Involved in the unfolded protein response.
Selenoprotein W	Needed for the muscle metabolism.
Selenophosphate synthetase	Synthesize selenophosphate.

Selenium metabolism in human body

Dietary food provides Se source for human beings, the absorption of selenium in intestine as shown in **Fig I-1**, Se(IV)cross the intestinal barrier by paracellular pathway. Organic Se such as SeCys, SeMet through the transcellular pathway. Likewise, Se(VI) follows the same pathway as organic Se forms, subsequently is reduced into selenite. Following, all the Se species metabolized into hydrogen selenide (HSe^-) [20], which is the precursor of selenoproteins. Not all the hydrogen selenide is used for selenoprotein synthesis, thus, the excess is excreted as selenosugars, Trimethylselenonium ($TMSe^+$) in urine, Dimethylselenide (DMSe) and Dimethyldiselenide (DMDSe) in the breath. The bioavailability of Se is intimately related with its chemical form. In general, organic Se species are more bioavailable than inorganic ones, for which the bioavailability can reach values up to 80-90%, whereas only 20% of inorganic Se is able to reach to the circulatory system in the human body [21,22].

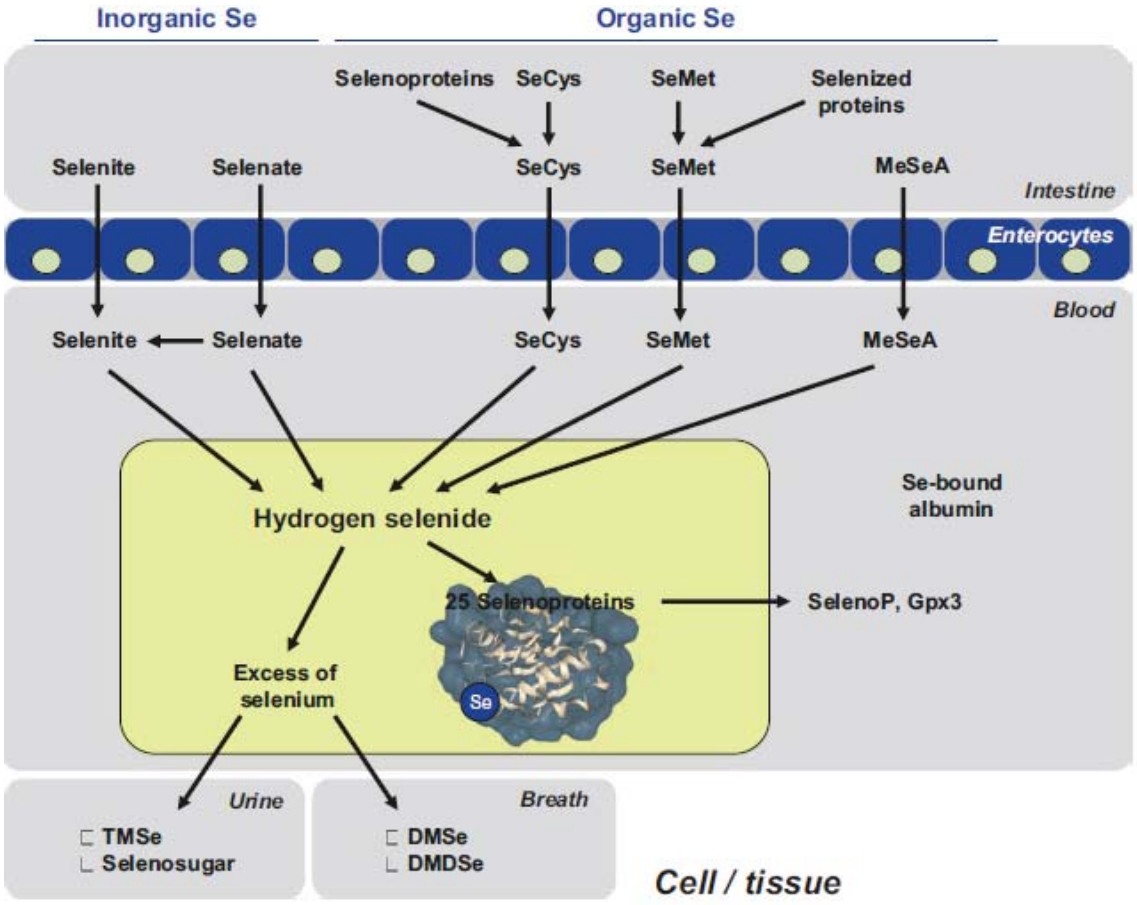

Figure I-1. Selenium metabolism in human cell or tissue [23]

The lack of selenium problem for human

The adult should have at least 40 μg/day of Se to support the maximum expression of Se enzyme and 300 μg/day to reduce the cancer risk [24]. However, 500-1000 million people in the world are suffering the Se deficiency, which is approximately 15% population in the world with insufficient Se levels in their blood [24–26]. Selenium is distributed unevenly across the planet; its concentration is from 0.005 to 3.5 $mg.kg^{-1}$. Selenium deficiency has been reported in all regions of the world including UK, Finland, Demark, New Zealand, Australia, India, Denmark and some area in China, with a low concentration of Se in soils, therefore, their food system with the daily Se intake is less than 55 $\mu g.g^{-1}$. The daily Se intake between 55-100 $\mu g.g^{-1}$ are those regions suffering low Selenium such as certain

regions of Switzerland, Korea. Oppositely, part of Iceland, Colombia and Canada are seleniferous [27], with a daily Se intake between 100-200 $\mu g.g^{-1}$. (**Fig I-2**)

Some countries like Finland and Denmark have been using Se-containing fertilizers to amend the deficiency of Se in the soil. It is not apply widely around the world since the pH, moisture and microorganism present in the soil are quite various in different regions [27]. The common inorganic anions of Se are SeO_3^{2-}, SeO_4^{2-}, $HSeO_4^-$, $HSeO_3^-$. SeO_3^{2-} and $HSeO_3^-$ (pKa=7.3)are favored forms in well-drained mineral soils with a neutral to acidic pH[28]. Besides, SeO_4^{2-} and $HSeO_4^-$ (pKa =1.9) are the predominant forms in neutral and alkaline soils or under oxidizing conditions.

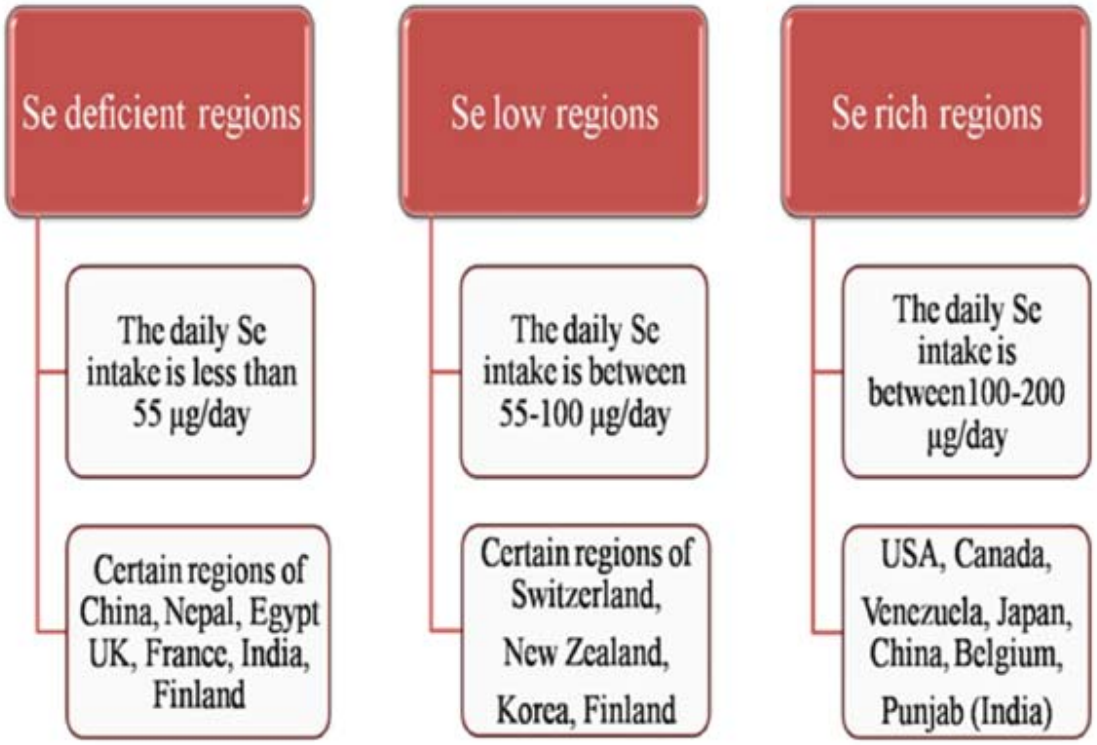

Figure I-2. Outline of occurrence of Se in different regions of world as Se-deficient, Se-low and Se-rich regions [29].

1.3 Selenium supplement functional food

Soil Se levels generally reflect its presence in food and the Se levels in human population. While Se bioavailability in human body is affected by Se speciation intake. Selenium speciation variety present in the food resource due to the different biosynthetic pathways involved in Se assimilation by plants and how these species are metabolized in animal.

Se speciation

Selenium bioavailability depends on the Se species. Normally, organic Se is less toxic than inorganic form in mammals, for which the bioavailability can reach values up to 80-90%, whereas only 20% of inorganic Se is able to reach to the circulatory system in human body [21,22]. Selenium speciation related to present study as shown in **Fig I-3**.

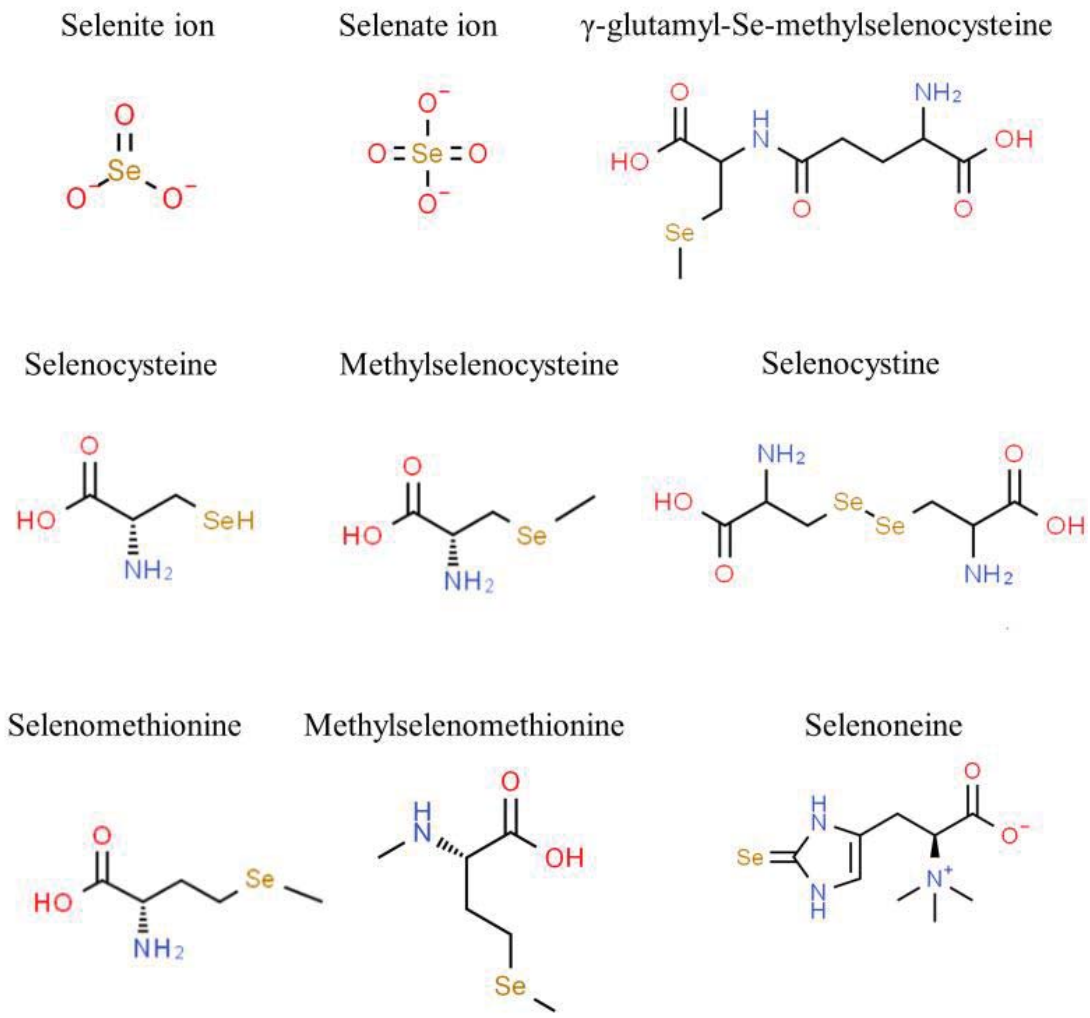

Figure I-3. Se speciation related to present study [30].

Selenium speciation found in daily food

Selenium speciation variety present in the food resource due to the different biosynthetic pathways involved in Se assimilation by plants and how these species are metabolized in animal [20].

Selenomethionine (SeMet): Found in plant, especially cereals, selenium enriched yeast. It is incorporated non-specifically into human protein instead of methionine.

Selenocysteine (SeCys): Found in animal food, as an active center exits in selenoprotein in human.

Selenoneine (2-selenyl-N α,N α,N α-trimethyl-L-histidine)**:** Found in fish such as tuna [31].

Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine: Found in plant, selenium enriched yeast, garlic, onion. It is metabolized to methylselenol, which is thought to have anticancer effects.

Sodium selenite and selenate: Dietary supplement, plant sources.

Daily selenium sources

Selenium is the rare nonmetallic element distributed unevenly on the Earth's crust. The concentration of Se in rocks depends on rock type, sedimentary rocks are found higher Se than the igneous rocks [32,33]. Se in soil is released by the natural processes such as weathering, volcanoes and as well human activies [34] (**Fig I-4**). The Se in the atmospheric is emitted by the marine biogenic Se, metal refining and coal combustion.

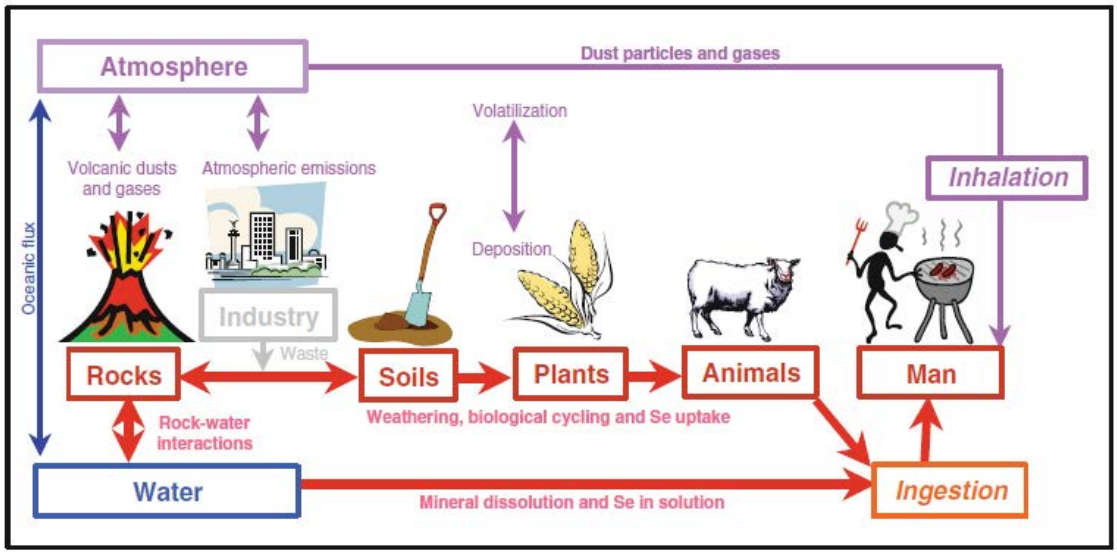

Figure I-4. Simplified schematic diagram of the cycling of selenium from environment to man. The main geochemistry and health pathways are shown in red/thicker arrows [13]

Plant can absorb Se(IV) and Se(VI) and transform them into organic forms, which are consumed by animals. Finally, selenium goes to human body via the food chain. As a result, the Se levels in soils are the key point of Se levels in plants. Similarly, the Se content of animal products reflect the Se levels in their consumed diet.

Although soil Se levels can react Se level in food chain directly, the food protein is another factor affecting the Se content in food because Se can replace S in the aminoacids and incorporated in the protein.

The protein enrich food such as egg, chicken, meat contain high level of Se ranging from 87.6 to 737 $ng.L^{-1}$ [35]. The milk product by different animals like human, sheep and cow. The Se content in the cow milk is around 20 $ng.L^{-1}$, which is lower than the milk in human and sheep. Fruits and vegetables contain low protein fraction resulting in the low Se concentration [36] (1.7-24.9 $ng.g^{-1}$) except mushrooms that has been found 1340 $ng.g^{-1}$ Se

in the report [37]. Nuts, cereals and by-products contain an important level of Se with a large range between the different species.

The role of plant on Se speciation (metabolism) and the meaning for human

Selenium bioavailability depends on the chemical form [38]. The data from report of the US Food and Nutrition Board [39] deem that dietary selenium is highly bioavailable: organic Se like SeMet and SeCys are absorbed very well, over 90%. Whereas, about 100% of selenate is absorbed, but a statistical faction is lost in the urine. Over 50% of selenite is absorbed and better retain than selenate. In general, organic Se species are more bioavailable than inorganic ones, for which the bioavailability can reach values up to 80-90%, whereas only 20% of inorganic Se is able to reach to the circulatory system in human body [21,22].

Plant is a favorable media to transform the Se inorganic species present in the substrate into seleno-aminoacids, which are the desired Se forms for animal and human intake. Therefore, the present of Se in our diet comes indirectly from the reservoir in soils, mainly via plants and/or meat consumption.

Plant Se metabolism largely follows S uptake and metabolic pathways non-specifically [40]. In fact the uptake of Se(VI) via sulphate transporters has been well documented [41,42]. Whereas, the mechanism involved in the uptake of selenite still remain unclear, earlier studies suggest that Se(IV) uptake by plant root is through passive diffusion[43,44]. Oppositely, Terry et al.[41] insist that Se(IV) uptake is not mediated by membrane transport. Furthermore, Li et al [45] has provided physiological evidence that Se(IV) can be taken up by phosphate, Hopper & Parker [46] found that Se(IV) uptake is to be depressed by phosphate in long-term hydroponic experiments.

Once the plant has taken up inorganic Se by root cells, Se(VI) is reduced to Se(IV) via ATP-sulfurylase, which is a rate-limiting step for Se(VI) assimilation in the plant. Then Se(IV) is reduced to selenide with enzymatic or without enzymatic participation.

Then selenide is incorporated into SeCys via the O-acetylserine. SeCys can be converted to SeMet via the methionine cycle, with Se-cysthationine and Se-homocysteine as the intermedia [47]. The amino acids SeMet and SeCys can be methylated to form methyl-SeMet and methyl-SeCys, respectively [48], which are the precursors for the volatile Se species DMSe and DMDSe (**Fig I-5**).

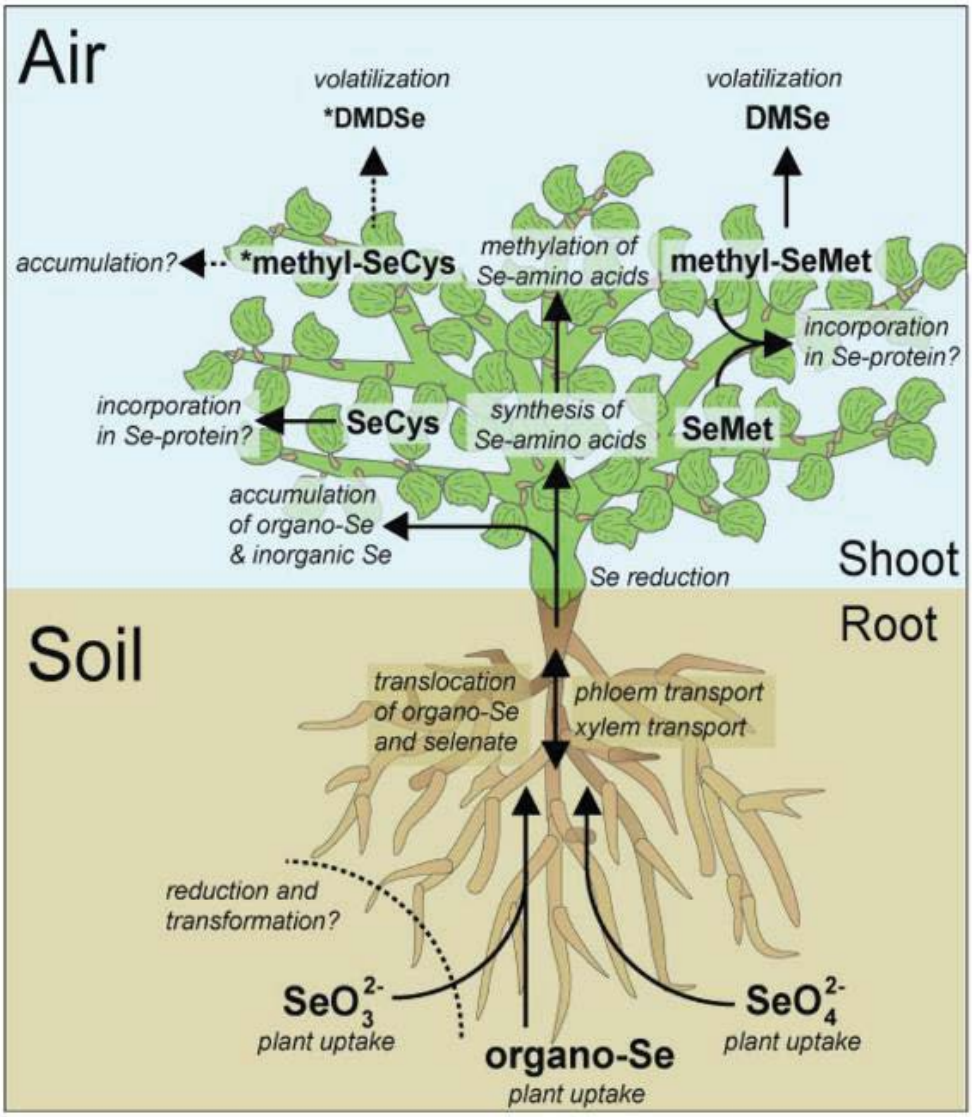

Figure I-5. Schematic diagram of the biochemical reactions in the uptake and metabolism of Se in plant [40,49]

1.4 Selenium for the plants

Plant can take up and metabolize Se due to the similar chemical properties between Se and S. Selenium atom can replace S atom in amino-acids and incorporate them into Se-proteins, which led to the disruption of structure and function of those proteins. Plants have different ability to tolerate Se with accumulation values that range from < 100 $mg.kg^{-1}$ to > 1.000 $mg.kg^{-1}$.

Benefit

Although Se is not an essential element for most of the plants, many studies have shown that Se at low concentration can protect plants form different abiotic stress such as cold [50], drough [51], desiccation [52], and metal ion stress [53]. Some reports also proved that Se can increase the yield in *Cucerbita pepo* [54], and protect the plant from insects [55]. The application of Se can interact with heavy metals, such as Hg [56], Cd [57], potential in reducing the toxicity of heavy metals in plant.

Toxicity

The main reason of Se toxicity on plants is the replacement of Met and Cys by SeMet and SeCys respectively in the amino acids to be nonspecifically incorporated into Se-proteins, which led to disruption of their structure and function [58]. The other reason of Se toxicity on plants is the oxidative stress promoted at high doses of Se [29] that acts as pro-oxidant and generates reactive oxygen species (ROS). Mroczek [59] has reported that root of *Vicia faba* exposed to lower doses of Se could decrease ROS levels better than at higher ones.

The limit of high concentrations and low concentrations of Se depends on the plant species and it is not unified. Plants have been classified as hyperaccumulators, secondary-accumulators and non-accumulators (as shown in **Fig I-6**), which have totally different tolerance to Se accumulation. The hyperaccumulators have > 1.000 mg $Se.Kg^{-1}$ DW in their cells, such as *Stanleya and Astragalus*. The Se species in hyperaccumulators are Methyl-SeCys and Methyl-SeMet, which are detoxified by methylation of SeCys and SeMet. These forms of Se do not interfere with S metabolism [40]. It has been suggested that Se is essential for hyperaccumulators, since they have strong positive growth effects in its presence. It was suggested that Se hyperaccumulators have Se-specific metabolism

because it they can distinguish S and Se [60]. Secondary-accumulators can accumulate 100-1000 mg Se.Kg^{-1} DW in their cells without signs of toxicity, like *Brassica and Broccoli*. Besides, grasses and crops are non-accumulators and can accumulate less than100 mg Se.Kg^{-1} DW.

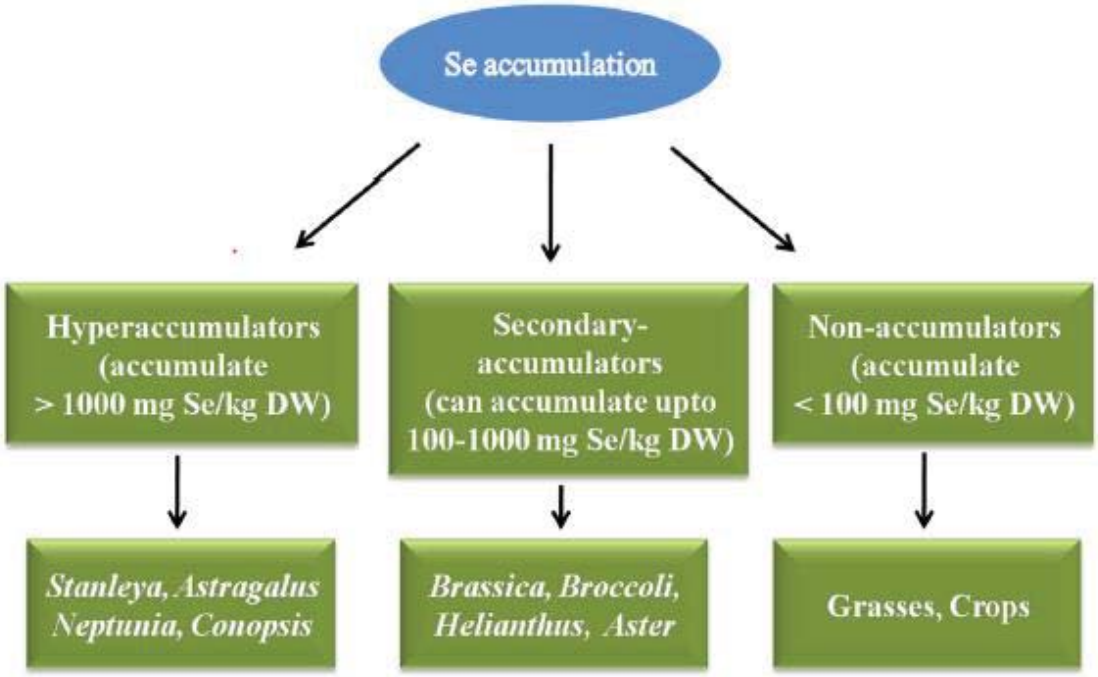

Figure I-6. Classification of plants depending on Se accumulation as hyperaccumulators, secondary-accumulators and non-accumulators [29].

The strategy for enhancing Se accumulation and tolerance in plant

In order to enhanced Se tolerance and accumulation, genetic engineering has been used as a strategy. Several studies proved that overexpression of the key enzyme ATP sulfurylase which reduces Se(VI) to Se(IV), resulted in the enhancement of Se accumulation [61]. Overexpression of SeCys methyltransferase from *A. thaliana and B. juncea* enhanced the nontoxic Se form of methyl-SeCys [62]. Selenocysteine lyase was overexpressed in *A. thaliana and B. juncea* can reduce the incorporation of Se into proteins because the enzyme breaks down SeCys into alanine and elemental Se (Se^0) [63,64].

Agronomic biofortification is the other strategy that can be used to enrich agricultural foods with Se, and certain other nutrients as well [65,66] . It is an economical safe agricultural technique to increase Se in edible plant parts. The objective of Se fertilization is to increase the concentration and bioavailability of Se in soils, which is a process focusing on nutrients provided when plants are growing. Although mineral elements can be present in the soil in bioavailable forms, several soil factors such as pH, redox conditions, cation exchange capacity and organic matter content have a great influence on their absorption by plants. Thus, foliar applications of soluble inorganic fertilizers are made as well to avoid these effects.

Selenium and wheat

Wheat is a worldwide consumed crop being used for human food and livestock feed in Australia, most of Europe, Northern Asia and Northern Africa (**Fig I-7**). The wheat flour used to meet consumer demands for bread, noodles, biscuits, cakes, paste, etc. Wheat provides high content of starch, about 60-70%, 8-15% of protein, variety of minerals, and vitamins to human diet [67]. There are more than 25.000 types of wheat that are adapted to different temperate environments. Wheat can exceed 10 tones ha^{-1}, rather than the other temperate crops under the appropriate growth condition [29].

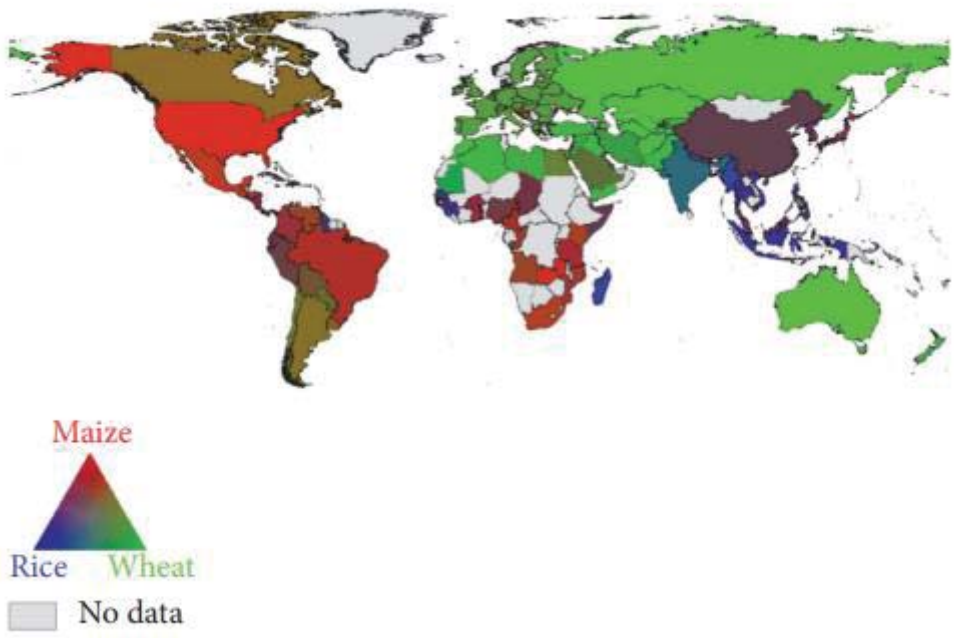

Figure I-7. Global major grain consumption map [68]. Colors represent the relative consumption of corn, rice and wheat.

Wheat are selected as the most important source of Se since it is the most efficient accumulator of Se within the common cereal crops (wheat > rice > maize > barley > oats) [69]. In UK, wheat has been chosen as the target crop for agronomic biofortification to increase the dietary Se intake [70]. Selenium enriched wheat in Nawanshahr-Hoshiarpur region in Punjab (India) [71] has high concentrations of Se ranging from 29 to 185 $\mu g.g^{-1}$. The regular consumption of such wheat can produce Se toxicity, but can be used as Se supplement in diet in low Se areas [29].

1.5 The role of plant biostimulants

Plant biostimulants based on natural substances have received crucial attention by both scientific community and commercial enterprises especially in the last two and a half decades. These products are used to improve nutrient efficiency, abiotic stress tolerance and crop quality.

The definition of plant biostimulants

The industrial definition of biostimulants was originally proposed in 2012 and stated: "Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides". The clearest and most concise way to describe biostimulants was proposed by du Jardin [72]: "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content". It is a kind of functional product between a fertilizer and a pesticide.

The biostimulant studied in this thesis is: a new composition agent, a plant anti-stressor and phyto-fitness, based on complex of hybrid hetero-polyoxometalates (containing molybdenum (Mo), tugsten (W), vanadium (V), silicon (Si), boron (B), phosphorus (P) and Se as well) of Keggin and Dawson structure with humic acids. Trace and micro-elements such as iodine (I), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), zinc (Zn) and copper (Cu) in ionic forms are also part of its composition, which is prepared by one-pot/domino oligo-condensation reaction of salts of the oxo acids above mentioned and also with salts of humic acids in aqueous solution at elevated

temperature. Obtained water soluble concentrate is diluted by water to concentration of 10^{-4} to 10^{-6} %.

The function and type of plant biostimulants

There are several categories of plant biostimulants that have been studied widespread such as algal extracts [73], protein hydrolysates [74], humic and fulvic acids [75], and microorganisms [76] (plant growth-promoting rhizobacteria). The mechanisms of action among the different plant biostimulants are quite similar, promoting the plant growth via ROS scavenging, membrane stability and osomoprotection.

Macroalgae from algal extracts used as fertilizer have been applied for thousands of years [77]. The algal extracts as biostimulants are used to tolerate salinity, heat and drough stresses, and a recent work has focused on the ability to cope with chilling stress (reference needed). Plants under drought stress and treated with protein hydrolysates had higher fresh weight and water content [78]. Finally, humic acid treated plants accumulated more N, Fe, Mn, S in the roots and shoots [79].

Objectives

The main objective of this study is to obtain Se enriched wheat plants and grain overcoming the Se-induced stress on growth by applying a plant biostimulant based on a complex of hybrid heteropolyoxometalates of Keggin structure molecules mixed with humic acids, called “Phyto-Fitness” (BIO Fitos S.R.O., Czech Republic). Secondly, we want to evaluate the biostimulant effect on the Se speciation in wheat plant.

For these purposes wheat plants were grown hydroponically and exposed to different Se treatments (selenite, selenate and the mixture of both species) and different biostimulant application modes (foliar or through the root system) as well as at different wheat growth stages (tillering or heading stages).

Firstly, xylem sap is collected to investigate the different behavior of the two main inorganic selenium anions, selenite and selenate, and to investigate the effect of Se species on sap pH. In this study, xylem sap is used as an indicator of the capacity of nutrients translocation from roots to shoots. Furthermore, the investigation of application of biostimulants on enriched wheat plant are performed.

1) We investigate the short-term wheat plant on the response of biostimulant. Special attention is paid to plant biomass, plant growth hormones and the effect of Se on micro and macronutrient concentration as well as to the identification of the Se species present in different parts of the plant to evaluate a possible modification on the Se speciation by the biostimulant.

2) Furthermore, in order to maintain production yield and minimize both plant toxicity and optimize the total dosage of selenium supplementation, we monitor co-application of Se treatments and the biostimulant at different growing stages of wheat plants: at the heading stage (Head) or at the tillering stage (Tiller) until harvesting the grains at the senescence stage. We determine total Se content in grain, the grain biomass and we also use micro focus X-ray spectroscopy techniques (μ-XRF mapping and μ-XAS) to get a better insight in the distribution of minerals such as Mn, Ca, Mn, K etc, and speciation of selenium in different regions of wheat grain.

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Chapter II

Methodology

Methodology

Materials and chemicals

$CuSO_4 \cdot 5H_2O$ (≥98%), Cellulose, $CaCl_2$ (95%), KNO_3 (99%), $Ca(NO_3)_2 \cdot 4H_2O$ (99%), FeNa-EDTA (99%), H_3BO_3 (99%), $(NH_4)_6Mo_7O_{24}$ (99%), KOH (85.5%), KH_2PO_4, MES (2-morpholinoethanesulphonic acid) (99.5%), $MgSO_4 \cdot 7H_2O$ (≥99%), $MnCl_2 \cdot 4H_2O$ (99%), $ZnSO_4 \cdot 7H_2O$ (98%), Folin, Na_2CO_3, Galic acid(⩾ 98%) (VWR, Barcelona, Spain); Phyto-fitness (BIO Fitos S.R.O., Czech Republic).

HOAc, MeOH, 2-Propanol, (VWR, Barcelona, Spain); Na_2SeO_3 (AMRESCO, Barcelona, Spain); Na_2SeO_4 (FLUKA, Barcelona, Spain); 3-Indoleacetic Acid (IAA) and Indole-3-Butyric Acid (IBA), IAA (IAA-d5 98 atom % D), Seleno-L-methionine, Seleno-L-cystine and Se-(Methyl) selenocysteine hydrochloride (Sigma-Aldrich, Madrid, Spain). All the reagents used were of analytical grade.

Wheat plant culture design

Culture conditions and Hydroponic culture

Wheat (*Triticum aestivum L cv Pinzon*) seeds (Fitó S.A., Spain) were germinated on moist filter paper for 5 days at 25 ℃ in the dark. Seedlings were precultured in ½ strength Hoagland's nutrient solution[1] (**Table II-1**) (3 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 60 µM FeNa-EDTA, 2 µM $MnCl_2 \cdot 4H_2O$, 3 µM H_3BO_3, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 2 µM $ZnSO_4 \cdot 7H_2O$, 1 µM $CuSO_4 \cdot 5H_2O$) for two weeks in 6 L plastic pots (12 plants per pot) before applying selenium. The pH was buffered at 6.0 with 2 mM MES (2-morpholinoethanesulphonic acid) and adjusted with KOH (2 M).

The solution was aerated continuously and renewed every week. Plants were grown in a controlled-environment growth chamber with the following conditions: 8 h day/16 h night photoperiod with a light intensity of 320 $\mu Em^{-2}s^{-1}$.

Table II-2. Composition of ½ strength Hoagland's nutrient solution

Macronutrients	**Conc. of mother solution**	**Volume (mL) added from mother solution to final solution (60L)**	**Conc. of ½ strength Hoagland's nutrient solution**
KNO_3	1 M	180	3 mM
Ca $(NO_3)_2$	1 M	120	2 mM
KH_2PO_4	1 M	60	1 mM
$MgSO_4$	1 M	30	0.5 mM
FeNaEDTA	20 mM	180	60 µM
Micronutrients	**Conc. of mother solution**	**Volume (mL) added from mother solution to final solution (60L)**	**Conc. in the final solution**
$MnCl_2$	2 mM	60	2 µM
H_3BO_3	3 mM		3 µM
$(NH_4)_6 Mo_7O_{24}$	0.1 mM		0.1 µM
$ZnSO_4$ 7 H_2O	2 mM		2 µM
$CuSO_4$ 5 H_2O	1 mM		1 µM

Short-term plants experiment

In order to evaluate the effect of the plant biostimulant (Phyto-fitness, three batches of

plants were grown under the following conditions: No biostimulant (NB); foliar application (FA) of the biostimulant by spraying the product 100 times diluted on the leaves (single foliar application by spraying the product onto the leaves until every each leave is covered with dew, approximately 1.5 ml per plant); root application (RA) of the biostimulant through the nutrient solution, 1000 times diluted and renewed each time with the nutrient solution. After two weeks of pre-culture, plants were exposed to different selenium (Se) treatments: no selenium (0 µM); selenite (Na_2SeO_3, AMRESCO, America), selenate (Na_2SeO_4, FLUKA, Spain) and a 1:1 v/v mixture of both substances at concentrations of 10µM Se (**Fig II-1**).

After four weeks, plants were harvested, and root were desorbed with 10 mM $CaCl_2$ (VWR, Barcelona, Spain) cold solution to remove Se from the root apoplast. Leaves were washed with deionized water to remove residual sprayed plant biostimulant. Afterwards the plants were sectioned to separate root and shoot, and they were both lyophilized until further processing or usage. Dry root and shoot were weighted using an analytical balance.

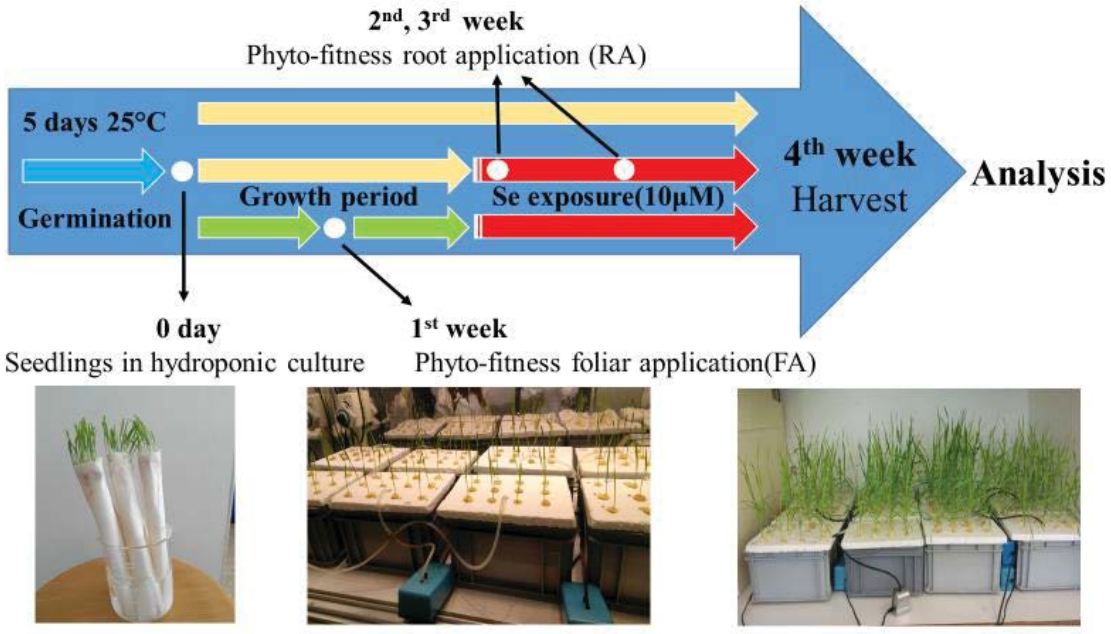

Figure II-8. Schematic of short-term plants

Long-term plants experiment

In order to evaluate the effect of the plant biostimulant on the Se uptake and accumulation in the plant, two batches of plants were grown in the presence (FA-foliar application) or not (NB-No biostimulant) of the biostimulant. The foliar application of the biostimulant was done by spraying the product 100 times diluted on the leaves. Moreover, the plants were exposed to different Se treatments: no selenium (0 µM); selenite (Na2SeO3, AMRESCO, America), selenate (Na2SeO4, FLUKA, Spain) and a 1:1 v/v mixture of both substances at concentrations of 10 µM Se.

In order to minimize both plant Se-induced toxicity and the economic cost of Se supplementation, one of the batches of plants was treated, as above mentioned, from the tillering stage (Tiller) and the other from the Head emergence stage (Head) and both maintained until the grain became mature. Afterwards, plants and grains were harvested and kept until further analysis (**Fig II-2**)

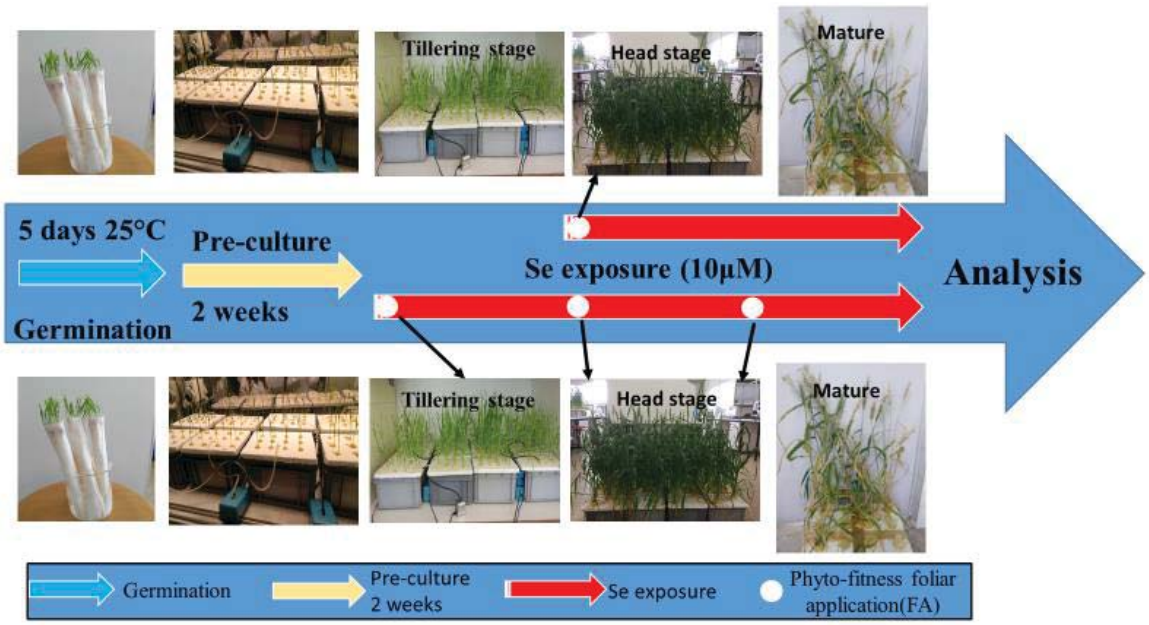

Figure II-2. Schematic of long-term plant experiment

Xylem sap experiment

Wheat plants were germinated and precultured as mentioned above for 1.5 weeks. Four batches of plants were grown under the following conditions: no selenium (0 µM); selenite, selenate and a 1:1 v/v mixture of both substances at concentrations of 10µM Se. After another 1.5 weeks, shoots were cut with a razor blade just 1cm above the root system which was connected to a proper size of PVC flexible clear pipe and sealed with parafilm. After 12 hours in darkness, xylem sap was collected in an Eppendorf. The aliquots were frozen immediately after pH measurement and stored at -20℃ for the subsequent determination (**Fig II-3**). Afterwards, the roots, shoots and a sample of the nutrient solution with and without plants were storage for further processing.

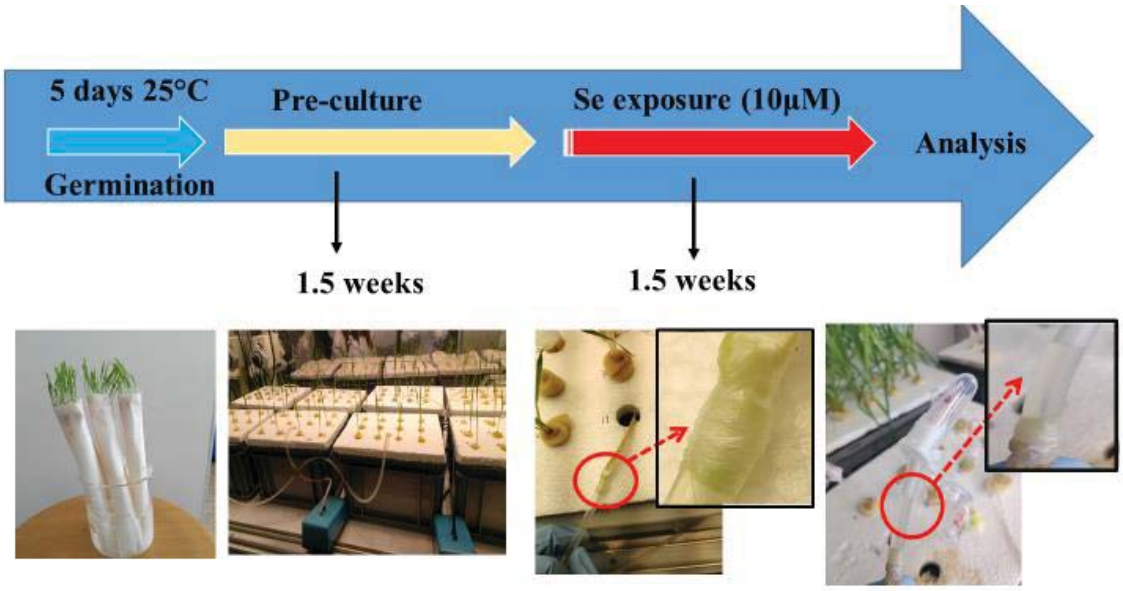

Figure II-3. Scheme of the xylem sap collection experiment.

Analysis and measurement

Total Se and mineral nutrient analysis

Powdered plant samples (n=4) were predigested with HNO_3:H_2O_2 (7:3, v/v) overnight then digested in hot block (Charleston, South Carolina, US) at 110℃ for 2h. Mineral nutrient concentrations were analyzed by ICP-MS (Perkin Elmer Optima 8300, Barcelona, Spain) and ICP-OES (Perkin Elmer Nexton 350D, Barcelona, Spain). Blanks were included in each batch of samples for quality control (**Fig II-4**)

Figure II-4. Part of equipment used in the study

Hormone extraction and measurement

The endogenous growth plant hormones, 3-Indoleacetic Acid (IAA) and Indole -3-Butyric Acid (IBA), were extracted and purified as modified by Llugany [2]. As shown in **Fig II-5**, briefly, 250 mg of fresh material was milled in an ice-cold mortar with 750 μL extraction solution constituted by MeOH: 2-Propanol: HOAc (20:79:1 by vol.). Then, the supernatant was collected after centrifugation at 1000 g for 5 min at 4 ℃. These steps were repeated two more times and pooled supernatants were lyophilized. Finally, samples

were dissolved in 250 µL pure MeOH and filtered with a Spin-X centrifuge tube filter of 0.22-µm cellulose acetate (Costar, Corning Incorporated, New York, USA). Hormone quantification was done using a standard addition calibration curve spiking control plant samples with the 2 standard solutions of IAA and IBA ranging from 50 to 1000 ppb and extracting as described above. Deuterated IAA (IAA-d5 98 atom % D) at 30 ppb was used as internal standard in all the samples and standards and all of them were purchased from Sigma-Aldrich, Spain. Plant hormones were analyzed by LC-ESI-MS/MS system in multiple reaction monitoring mode (MRM) according to Segarra[3]. First hormones were separated using HPLC Agilent 1100 (Waldrom, Germany) on an Acquity UPLC BEH C18 2.1 x 100 mm ID, 1.7 µm column (Waters, USA) at 50 °C at a constant flow rate of 0.8 ml min-1 and 10 µl injected volume. The elution gradient was carried out with a binary solvent system consisting of 0.1% of formic acid in methanol (solvent A) and 0.1% formic acid in milliQ H_2O (solvent B) with the following roportions (v/v) of solvent A (t (min), %A): (0, 2) (0.2, 2), (1.6, 100), (2, 100), (2.1, 2) and (3, 2).

MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, Ontario, Canada). All the analyses were performed using the Turbo Ionspray source in negative ion mode for IAA and the respective deuterated standard and positive ion mode for IBA.

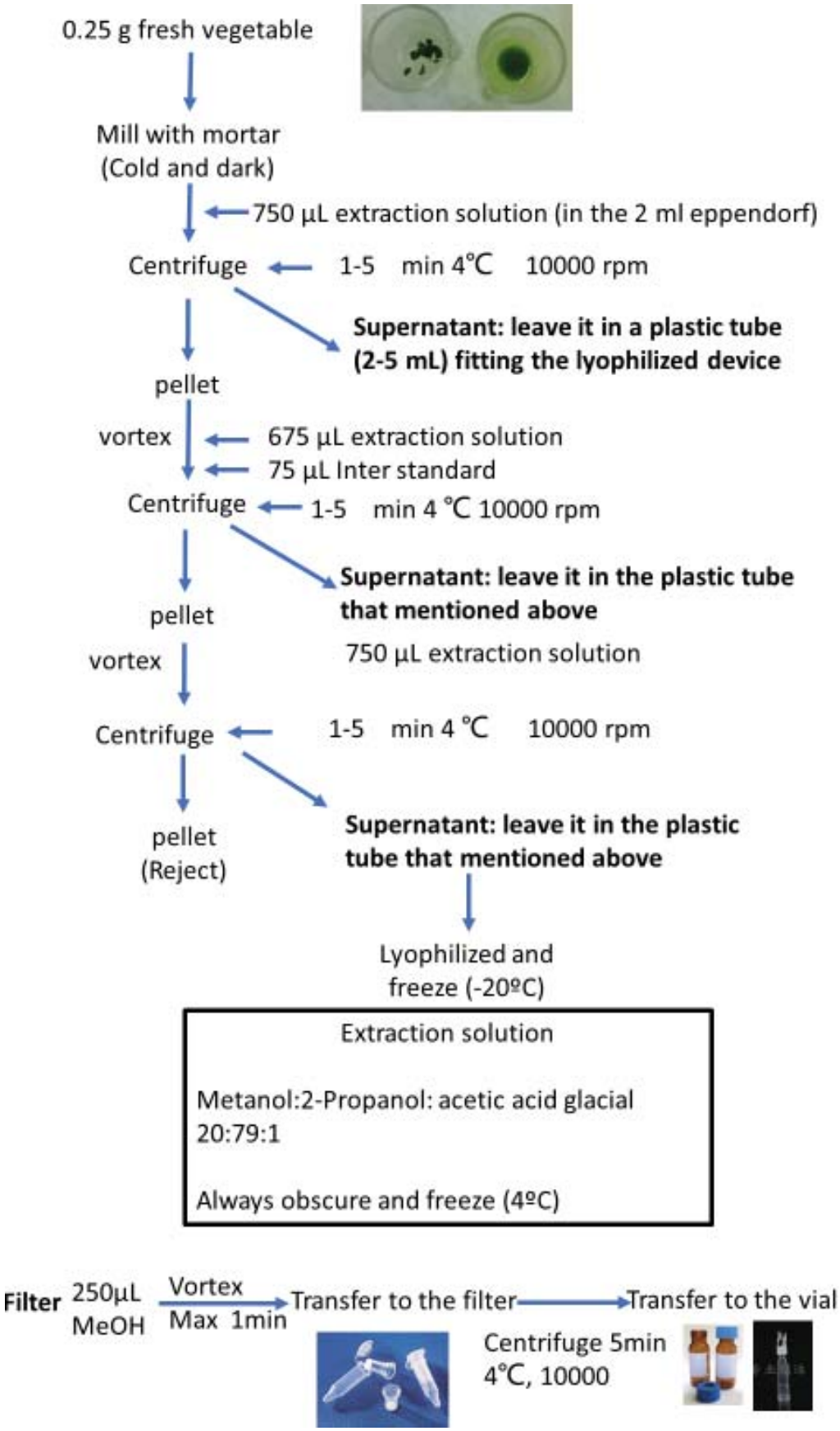

Figure II-5. Scheme of the hormone extraction procedure.

Titrator experiment (Fig II-6)

The titration curve was measure by automatic titrator (HANNA, HI931, Barcelona, Spain).

Analyte of Hoagland solution: consist of 2 mL Hoagland solution+8mL 0.1M KCl, titrant dosing type is linear-50 μL, time interval is 40 sec, under time increasing mode.

Analyte of xylem sap: consist of 0.2 mL xylem sap+0.8 mL 0.1M KCl. Titrant dosing type is linear-10 μL, time interval is 40 sec, under time increasing mode.

Titrant: 0.1 M HCl or KOH dissolved in 0.1 M KCl instead of water

Figure II-6. Image of titrator used in this study.

Synchrotron based X-ray Absorption Spectroscopic measurements.

Synchrotron technique (Fig II-7)

A synchrotron radiation as an important experimental tool has several important properties including high intensity, stability, broad spectral range, high polarization,

pulsed time structure, natural collimation. Electrons are produced by electron gun and accelerated in the linac and booster ring, to achieve the required energy. Then electrons are travelling at speed close to light in storage ring which consists of a roughly circular ring of magnets, high vacuum environment, insertion devices, wiggler etc. Tangential beam channels or ports attached to the ring permit the synchrotron radiation to enter the experimental hall.

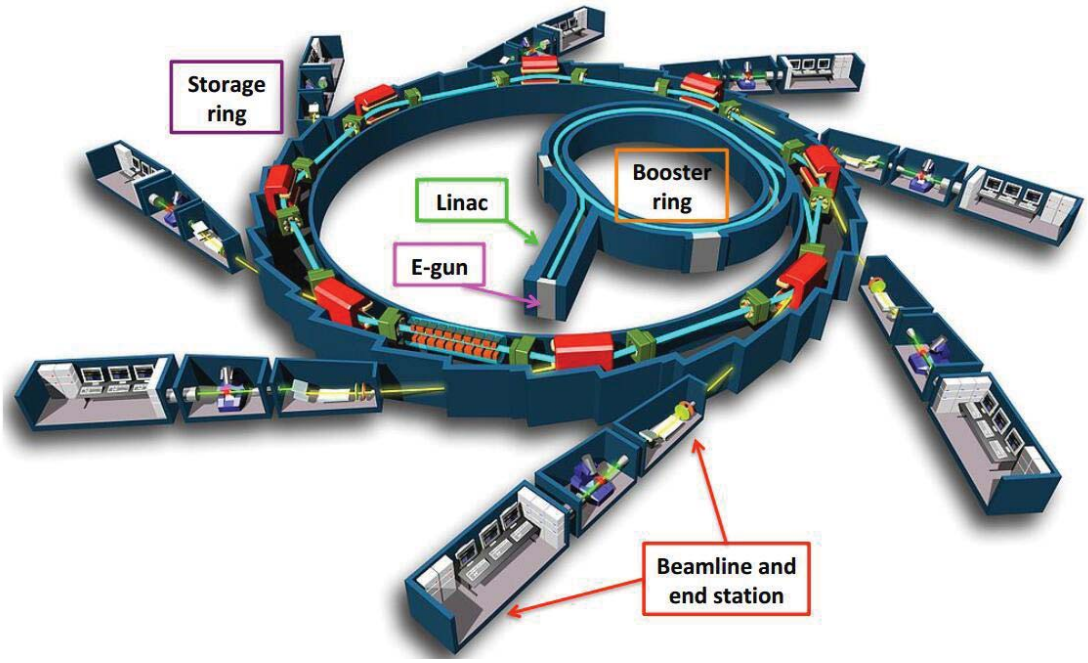

Figure II-7. The layout of synchrotron[4].

X-ray absorption spectroscopy (XAS) and X-ray fluorescence spectroscopy (XRF) theory

XAS and XRF are inner shell spectroscopies that an X-ray interacts primarily with a deep-core electron rather than with a valence electron (**Fig II-8a).** An incoming photon interacts with a 1st electron that is excited for a K-edge spectrum. Then the deep-core

electron is promoted to some unoccupied state above the fermi energy, propagates away, and leaves behind a core-hole. There are two main mechanisms for the decay of excited atomic state following an X-ray absorption event. The one shown in **Fig II-8b** is X-ray fluorescence, in which a higher energy core-level electron fills the deeper core hole, ejecting an X-ray well defined energy. Alternately, the energy from the higher lying electron can be used to emit an Auger electron (**Fig II-8c**). The use of fluorescence is more common used to measure the absorption coefficient μ..

XAFS can be measured either in transmission or fluorescence. Each element has a characteristic set of excitation and fluorescence. When the incident X-ray energy E is larger than the binding energy of a core-level electron, there is a sharp rise in absorption. The peaks, shoulders, and other features near or on the edges are known as X-ray absorption near edge structure (XANES). The ejected photos-electron can scatter from neighboring atoms. The gradual oscillations above the edge are known as extended X-ray absorption fine structure (EXAFS). Here, in our study, we mainly study XANES.

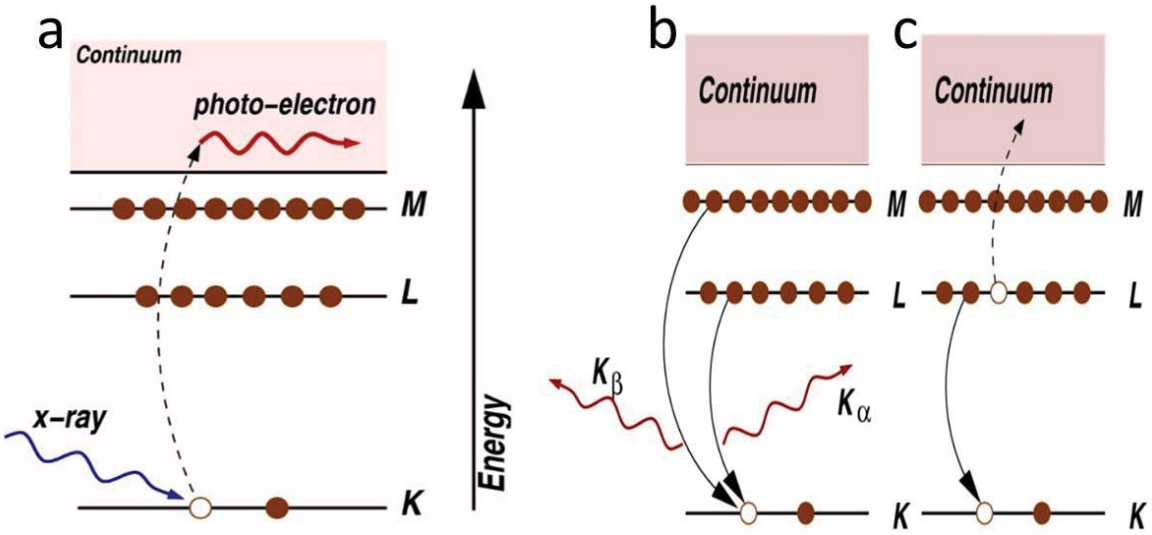

Figure II-8. The photoelectric effect, in which an X-ray is absorbed and a core level electron is promoted out of the atom [5].

XANES interpretation

XANES (**Fig II-9**) contains the following parts:

Pre-edge: Region before the edge where the incident energy is not enough to excite the desired photos-electron

E0: The inflection point in the absorption edge. Its position depends on the electronic density surrounding the studied atom.

Edge: The main rising part of XAS spectrum

Near-edge: Characteristic features above the edge

White line: Large, prominent peak just above the edge, particularly in L or M edge spectra.

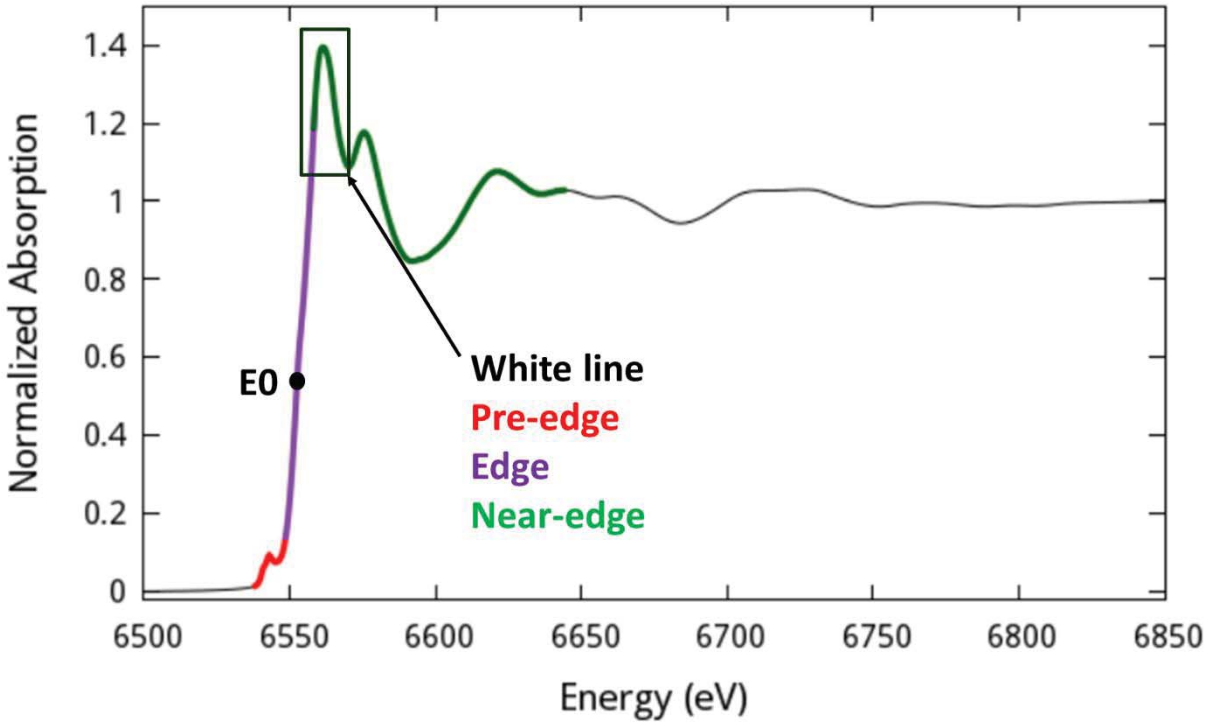

Figure II-9. Parts of a XANES graph/spectra.

One of the most powerful use of XANES data is fingerprint which simply identify by mean of a distinctive mark or characteristic. Further, Linear combination fitting (LCF) is used to get quantitative results from XANES, which interpret data by comparison with standards.

The element selectivity of X-ray absorption spectroscopy (XAS) provides a unique insight to determine the speciation of Se in biological samples with mild sample preparation. Alternative speciation methods based on the hyphenation of liquid chromatography and mass spectroscopy, such as HPLC-ICP-MS, requires sample pre-treatment which could modify the Se species under study and alter the results. The analysis of Selenium speciation in food is a challenging task; there are currently no methods that can reliably extract 100% of the selenium from foods without potentially affecting the species. Hence, XAS provides a direct insight of the chemical species in plant tissues.

Bulk analysis----ALBA synchrotron

Wheat samples

For the measurements, each sample was ground, and the powder was press into a pellet. XANES was collected at Se K-edge using QEXAFS scanning mode and the Si (311) monochromator crystal at CLAESS beamline, ALBA Synchrotron [6]. All the measurements were performed at liquid nitrogen temperature to avoid radiation damage on the samples. Due to the low concentration of Se in the plant samples, the measurements were performed in fluorescence mode using the multi-element Si drift detector with Xspress3 electronics (**Fig II-10**).

Selenium references

Additionally, sodium selenite, sodium selenate, seleno-L-methionine, seleno-L-cystine and Se-(Methyl) selenocysteine hydrochloride (Sigma-Aldrich, Madrid, Spain) were measured as Se references since they are the species expected to be present in the plant. Aqueous solutions of the appropriate concentration to obtain an optimum absorption jump (100-200 mM) were measured for each reference compound. The solutions were loaded into an in-house designed liquid cell with a 3mm transmission path and Kapton windows [7]. The references were measured in transmission mode using gas ionization chambers filled with the appropriate amount of nitrogen, argon and krypton to absorb 20% (I0) and 80% (I1 and I2) of the beam. Data normalization and analysis using linear combination fitting was carried out with Athena program of the Demeter software package [8].

Figure II-10. View of ALBA synchrotron and sample preparation.

X-Ray Fluorescence mapping (XRF)---- Diamond Light Source synchrotron

X-Ray Fluorescence mapping (XRF) offer powerful approaches for probing and mapping the *in-situ* distribution of a wide range of elements in plant tissue and organs.

Selenium References

Selenium references were the same as describe above. The appropriate mass of Se reference compound for getting an optimum absorption jump (calculated using XAFS mass software) [9] was mixed with cellulose (VWR, Barcelona, Spain) and pressed into a pellet instead of liquid cell methodology.

Grain samples

In order to obtain thin specimens, wheat grains were immersed in 4 ℃ MiliQ water. Then, humected grains were embedded in paraffin and cut by a microtome (Thermo Scientific HM 325) vertically. The ideal specimens were 60 μm thickness containing embryo, endosperm and outer layer.

Specimens were mounted on the top of carbon tape disk which was stuck on to a sapphire disk which were then glued on to the Al holder for the liquid nitrogen cryostat using super-glue. XAS and XRF mapping measurements on the grain sections were performed at beamline I18 of Diamond Light Source using a 4-element Si Drift fluorescence detector. Here, the distribution of Se, Zn, Cu, Fe, K, Mn, Ca in grain were obtained, with an excitation energy of 12677.0 eV (**Fig II-11**). Data normalization and analysis using linear combination fitting was carried out with Athena program of the Demeter software package [8].

Figure II-11. Images of microtome and ideal wheat specimens.

As shown in **Fig II-12**, specimens were mounted on the top of carbon tape disk which was stuck on to a sapphire disk which were then glued on to the Al holder for the liquid nitrogen cryostat using super-glue. XAS and XRF mapping of grain were performed monochromatic beam at beam I18 using 4 element Si Drift fluorescence detector, which is at Diamond light sources I18.

Figure II-12. View of diamond light source and part of samples preparation.

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 in order 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., St. Tulsa, OK, USA).

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Chapter III

Xylem sap analysis in Se-enriched wheat plants

Abstract

Wheat is a common cereal chosen by researchers for Se biofortification purposes. The target tissues are most roots, shoots and grains, but few studies focus on xylem sap. Xylem is the vascular lignified tissue specialized in the conduction from roots to shoots of a mixture of water with some inorganic (nutrients) and organic substances and signaling molecules, called xylem sap. In this study, xylem sap was collected from young wheat plants exposed to different Se species to analyze the total Se concentration, the sap pH and the nutrients translocated from roots to shoots. In addition, the influence of Se species was characterized by determining the acid-base behavior of the xylem sap.

We observed that micronutrients such as Zn, Cu and Mo decreased in concentration in the sap of plants treated with Se(IV) which is in accordance with the accumulation of these mineral elements in the shoots. To sum up, a quick and low-cost method based on acid-base titrations for basically testing the xylem sap response to the different Se species has been developed.

Key words: xylem sap, titrator, selenium.

Introduction

Selenium (Se) as an essential element is beneficial for human health, which is required for a variety of biochemical and physiological activities in human body, such as antioxidant defenses, immune function and formation of sperm [1]. The resource of Se supplement from daily food are diverse, largely required from edible plant. Selenite (Se(IV)) and selenate (Se(VI)) are the predominant inorganic Se forms taken up by plants. These inorganic forms are transformed by plants into organic ones that are assimilable for human organism [2]. Although Se is not an essential element for plants, Se can be taken up and assimilated by them as the chemical properties of Se are akin to that of sulphur (S). Once plants take up Se or nutrients via the root system, those nutrients will be transported to shoots immediately via the xylem.

Xylem is a conductive tissue in vascular plants, which transports water and some mineral elements from roots to leaves. Xylem sap acts as the blood in plant tissues carrying the essential nutrients to sustain plant growth, likewise photosynthesis products are allocated by phloem. Besides, xylem sap contains not only minerals and water, but also amino-acids, organic acids, sugars, alkaloids and signaling molecules [3,4]. The aerial part is dependent on the compounds that are taken up and/or produced by roots and distributed by the xylem network [5]. Those compounds are also involved in long-distance signaling in response to stress response factors [6]. These signaling molecules and the application of a simple and straightforward analytical titration technique of the xylem sap allow us to further insight into the plant response to Se enrichment process [7].

Many studies [8–10] have revealed that the Se(IV) and Se(VI) have different metabolic behavior during the process of uptake and mobility in plant. Several studies [8,11–13] have been focused on Se-enriched wheat, however, most of them were targeting on different

plant tissues such as, roots, shoots and grains, but few of them have focused on the xylem sap level.

Nevertheless, heavy metals, plant hormones and other nutrients present in the xylem sap of crops have been investigated. As for example, arsenic (As) speciation in wheat and rice was investigated during As uptake and translocation [14], ABA levels as mediator of shoot cytokinin oxidase activity in wheat [15], P transport [16], apoplastic pH changes [6], signaling molecules related to drought stress [17]. Although Li et al [8], have investigated total Se and Se speciation in xylem sap of wheat plants treated with 10 μM Se, the mineral element concentration and the signaling in sap are still vacant to be addressed.

In addition, the small soil zone in contact to plant root, called rhizosphere, is a dynamic micro-environment, which is not only in charge of supplying water and nutrients to the plant, but also of receiving the rhizodeposition's secreted by the root such as organic acids, amino-acids, phenolic substances, enzymes, sugars, etc [18] that can change its chemical, physical, and biological properties. Rhizosphere and likewise the nutrient solution in hydroponic cultures are thus affected by plant roots and their metabolic activities. It has been pointed out that under environment stress, plant roots would excrete more metabolites and some unknown substances [19]. Selenium as an abiotic stressing substance may affect the metabolites present in the nutrient solution, in roots and in the xylem sap. Thereby, we will take this fact into account analyzing samples of the Hoagland nutrient solution in presence and absence of growing plants.

Taking altogether into consideration, the present study is addressed to focus on the influence of the Se enrichment process on the xylem sap composition of young wheat plants. Such influence will be characterized by determining the acid-base behavior of the xylem sap from plants enriched with different Se species compared to non-exposed ones

(control) and the analysis of the mineral nutrient contents both in sap and shoot by ICP-MS.

Methodology

Plant material and xylem sap collection

Wheat plants were germinated and grown as described in the general methodology section. Four batches of plants were exposed for 10 days to the following conditions: No Se (0 μM); Se(IV), Se(VI) and a 1:1 equimolar mixture of both substances at concentrations of 10μM Se. Afterwards, shoots (1.0 cm above the roots) were excised with a razor and the remaining part was fitted to a short length PVC flexible tube to collect the spontaneous xylem sap exudate, which was removed after 12 hours by means of a pipette, placed in an Eppendorf tube, and immediately frozen at -20 ℃ after pH measurement (**Fig. III-1**). The shoots and a sample of the initial and the final nutrient solution were stored until further processing.

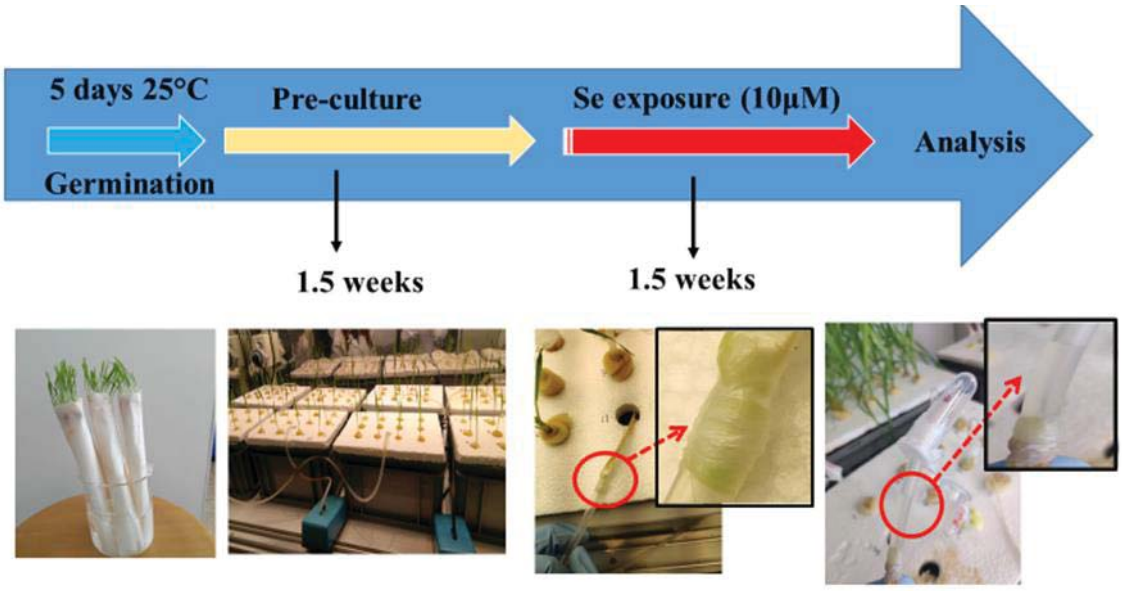

Figure III-1 Schematic representation of xylem sap collection from *Triticum aestivum*.

Total Se and mineral nutrient analysis

Powdered plant samples (shoot) (n=4) were predigested with HNO_3:H_2O_2 (7:3, v/v) (VWR, Barcelona, Spain) overnight then digested in hot block (Charleston, South Carolina, US) at 110 ℃ for 2 h. Mineral nutrients in shoots were analyzed by ICP-MS (Perkin Elmer Optima 8300, Barcelona, Spain). Blanks were included in each batch of samples for quality control. Xylem sap samples were just appropriately diluted.

Measurement of xylem sap pH

The pH value of the xylem sap collected from the different Se treatments was measured with a micro-pH electrode (HANNA, HI931, Barcelona, Spain). Three replicates were processed and measured for each Se treatment.

Acid-base titrations of Hoagland solution and xylem sap

The medium of analyte

Acid-base potentiometric titrations were carried out in aqueous 0.1 M KCl to keep the ionic strength constant and avoid the Na+ interference in pH measurements [20].

The medium of titrant

To determine whether the medium of titrant, between Milli-Q water and 0.1 M KCl, could influence the titrant results reflected on the shape of titrant curve, two groups of experiments were performed, 0.05 M HCl and 0.05 M NaOH as acid and base titrants respectively. Thus, samples (titrant) were dissolved either in Milli-Q water or 0.1 M KCl. Solution of analyte in each group was comprised of 0.2 mL 0.01 M HOAc and 0.8 mL 0.1 M KCl. The titration curves were measured by automatic titrator (HANNA, HI931, Barcelona, Spain). Titrant dosing type is linear-10 μL, time interval is 40 sec, under time increasing mode.

Selected pH interval for titrations was 3.0 < pH <12.0 by using either NaOH or HCl as respective titrants. All samples were titrated by adding first the acid titrant and after the basic titrant to verify the reversibility of the target samples. All the titrations were repeated 3 times for assessment.

Measurements of the initial and final Hoagland solutions and of the xylem sap by titrator

All samples were spiked with HCl to reach the acidic pH (approx. pH=3.0), subsequently, the NaOH was added to get the complete curve. Since these two curves were overlapped, therefore, only the NaOH curve was shown in the graph. General parameters in titration procedures are shown below.

Analyte of Hoagland nutrient solution: Consist of 2 mL Hoagland nutrient solution + 8 mL 0.1M KCl. Titrant dosing type is linear-50 μL, time interval is 40 sec, under time increasing mode.

Analyte of xylem sap: Consist of 0.2 mL xylem sap + 0.8 mL 0.1M KCl because of the insufficient volume of sap for analysis. Titrant dosing type is linear-10 μL, time interval is 40 sec, under time increasing mode.

Titrant: 0.05 M HCl or KOH dissolved in 0.1 M KCl instead of water.

Statistics

To check the reproducibility of the results, the entire experiment was repeated three times in different seasons; spring, summer and winter. The results given are means (n=3) with standard error (SE). All the data were checked for normality using Kolmogorov- Smirnov test and data not normally distributed were log transformed in order 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 done with Statistic software version 6.0 (StatSoft Inc., St. Tulsa, OK, USA).

Results and discussion

pH in xylem sap

Long distance signals from roots to shoots via xylem are important in plant responses to abiotic factors, such as Se-induced stress. The pH values of xylem sap were more alkaline around 7.5 compared to the pH of the initial nutrient solution which was 6.0 (**Fig. III-2**). Moreover, the Se species did not affect the pH in xylem sap.

The balance between cation and anion uptake has a considerable effect on pH in plant [6]. When anion uptake (e.g. NO_3^-) exceeds cation (e.g. K^+) uptake, stimulated organic acid

anion accumulation in the plant top, while only a small amount of HCO_3^- flux from root [21], the increased efflux of OH^- from cell may increase the pH of root apoplast [22,23]. Anions of organic acid (e.g. malate) may alkalize the pH of xylem sap more than inorganic anions due to its higher value of pKa. In addition, the alkaloids and alkaline amino acids are also needed to be considered as factors of increasing the Sap pH.

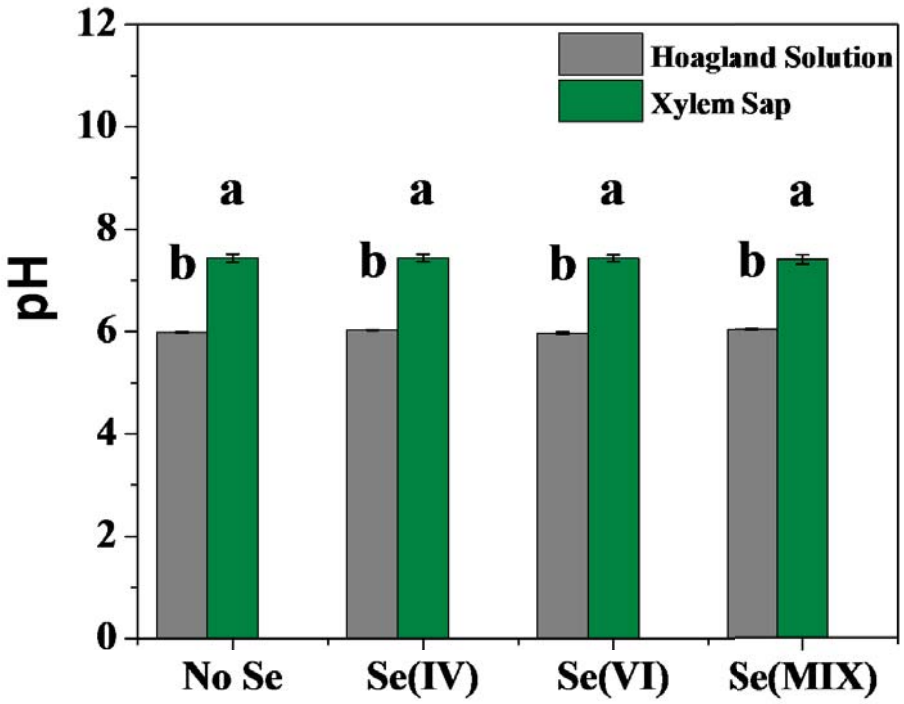

Figure III-2. pH values of the final Hoagland nutrient solutions and of the xylem sap collected from the corresponding *T aestivum* plants exposed to the different Se treatments.

Mineral nutrients in xylem sap

The concentration of the mineral elements in the xylem sap can be used as an indicator of the availability and translocation capacity of the nutrients from soils to roots and from roots to shoots. Studying xylem sap composition is a useful tool for investigating mineral uptake and allocation [24]. Mineral compounds loaded into the xylem remain for the most

part in the form of free ions and are carried in the transpiration stream to the shoot. From the apoplast or from the xylem, ions may be absorbed into the symplast, where selection of ions takes place.

The amount of total Se in the xylem sap of plants exposed to Se(IV) was lower than the Se concentration present in the nutrient solution. By contrast, the xylem sap of Se(VI) treated plants, had significantly higher total Se values than the corresponding nutrient solution. In addition, Se sap level in Se(MIX) plants was approximately the average between Se(IV) and Se(VI) group of plants (**Fig. III-3a**). The Se concentration in the xylem sap of Se(VI)-treated plants was 18.5 times higher than that of Se(IV)-treated wheat.

Due to the different mechanisms of Se uptake by roots, Se species show different mobility within the plant. Se(VI) is taken up actively via sulphate transporter as it has been well studied [9,25]. Unlike Se(VI), the mechanism for Se(IV) is still not fully understood. Some studies suggested that Se(IV) is taken up through passive diffusion through the plant root [26,27], this might be the explanation why total Se levels in the nutrient solution are much higher than they are in the xylem sap in this group (**Fig. III-3a**). Other studies suggested that selenite uptake an active process mediated, at least partly, by phosphate transporters [8]. Once Se(IV) taken up is quickly transformed to an organic form with a limited translocation to the shoot. By contrast, Se(VI) is highly mobile from roots to shoots via xylem transport with slow species transformation [8]. The main Se species present in the xylem sap is Se(VI), but small amounts of organic Se such as SeMet have also been reported [8,10].

Regarding to the micronutrients, we observed that the Cu concentration in the xylem sap was always significantly higher than in the nutrient solution in all the treatments

performed opposite to the values found for Zn and Mo. Moreover, the concentration of Cu, Zn and Mo in the xylem sap from plants exposed to Se(IV) were significantly lower than in the plants exposed to Se(VI) or to control conditions (**Fig. III-3 c, e, g**). Cu is actively taken up as Cu^{2+} by high-affinity copper transporters [28] as it is Zn [29] while the uptake of Mo is both passive and active.

Se species reduced the loading of Zn, Cu and Mo into the xylem **(Fig. III-3c, e, g)** when plants were treated with Se(IV). It indicates that Se(IV) plays more hazard role than Se(VI) at 10 μM. This could be due to a reduction in water content and thus in mineral compounds uptake. **Fig. III-3b, d, f, h** shows that the pattern of shoot element accumulation was similar to that of xylem sap elements concentration. The results suggest that translocation from root to shoot is a key process to control Se and element accumulation in wheat aerial part. The Cu, Zn and Mo concentrations in shoots are in agreement with the result from Łukaszewicz et al.[30] in garden pea. A reduction in the water content in roots was the attributed reason for the lower uptake and transport of ions, and an inhibition of the shoot dry matter.

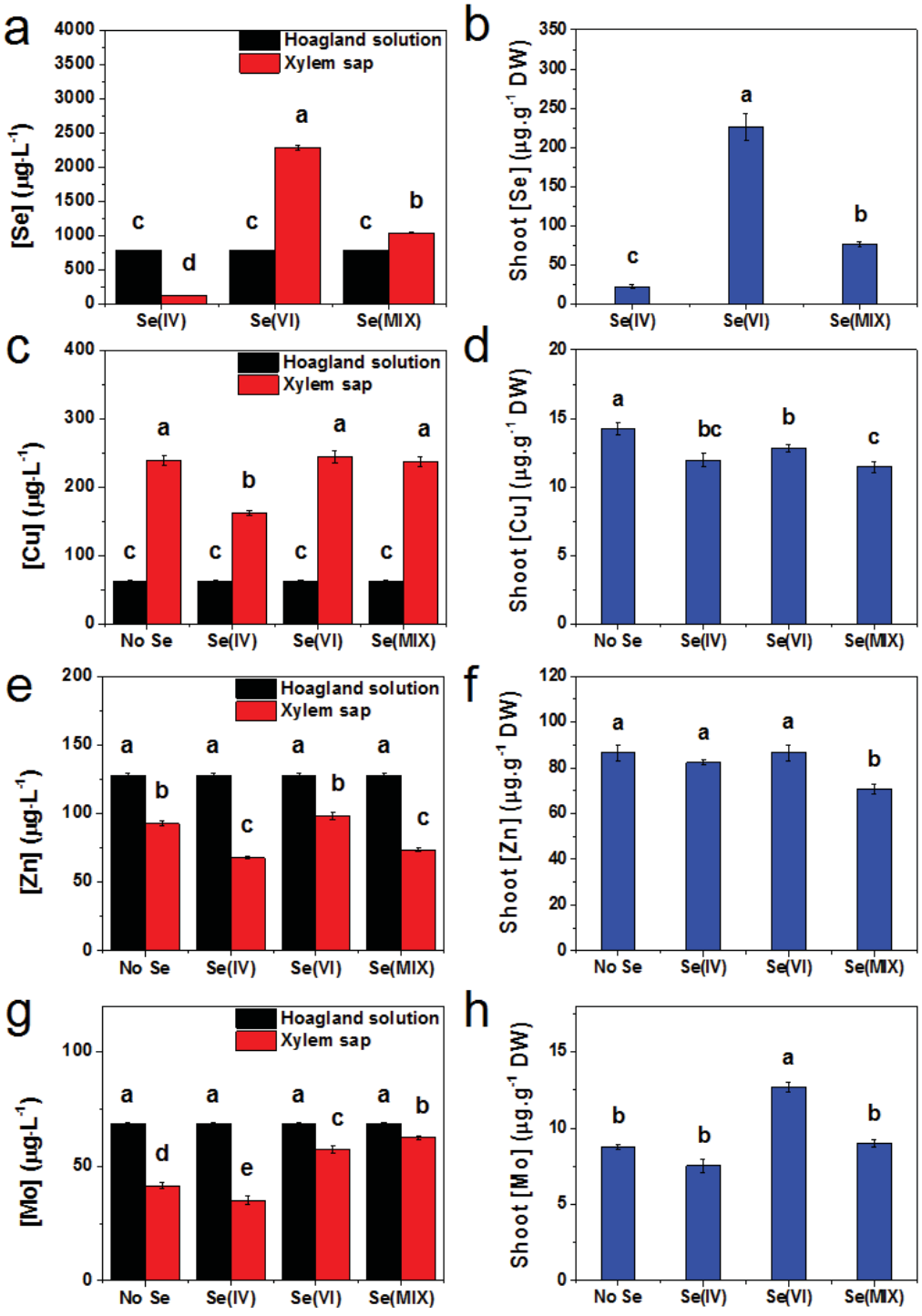

Figure III-3. Concentration of Se, Cu, Zn, Mo in the initial Hoagland nutrient solution and xylem sap (a, c, e, g) and in shoots of *T. aestivum* plants (b, d, f, h) grown under different Se species at 10µM. Results shown are means ± SE (n=3). Different letters indicate statistically significant differences among groups (LSD, P<0.05)

The signaling in Xylem sap investigated by titration curve

Here we will include further study on the signaling in xylem sap, though the pH in xylem sap is not affected by the Se treatment, it has been pointed out that Se assimilation in plants not only affect sulfur (S) but also nitrogen (N) metabolism [31], thus, the S- and N-secondary metabolites and the ratio of those component in sap might be vary with the presence of Se. We have tackled this possible effects by exploiting the acid-base properties of sap by implementing acid-base potentiometric titrations.

Our results are based on the analysis of the respective titration curves, e.g, pH of the analyte solution versus the volume of the titrant added as the titration progress. The shape of the titration curve is an experimental signal of the acid-base properties of respective xylem sap overall composition. By comparing those curves between different treatments, we could get basic information on the xylem sap composition.

The ionic medium and order of titrant added.

Titration curves were obtained in presence and in absence of constant ionic media (CIM), titrant dissolved in Milli-Q H_2O (**Fig. III-4a**) or 0.1 M KCl (**Fig. III-4b**), respectively. In absence of CIM, a gap between HCl and NaOH titration curves was found, while using CIM both curves overlap. This fact indicates the ionic strength variation during the titration, thus affecting the pH measurements in absence of CIM.

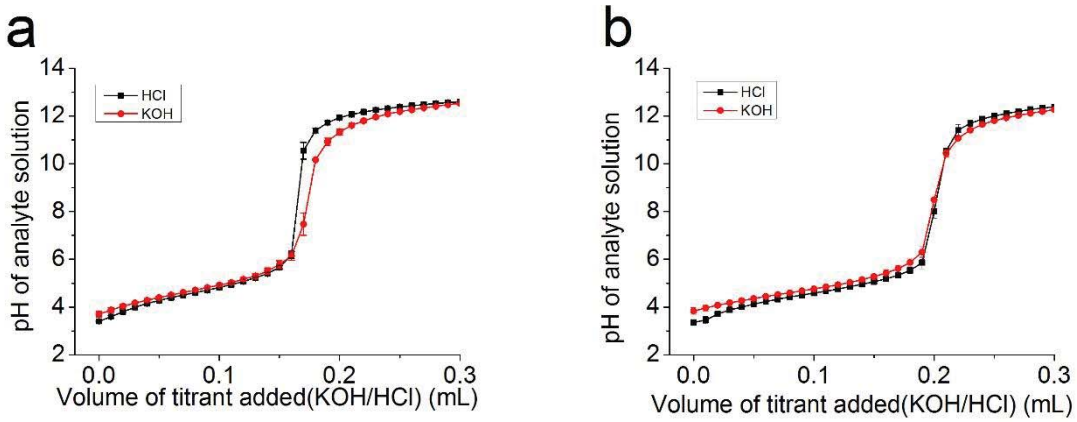

Figure III-4. Titration curve of the acetic solution. The titrant dissolved in Milli-Q water (a), the titrant dissolved in 0.1 M KCl, the same as analyte. Results shown are means ± SE (n=3).

Response of glass electrode to the titrant

Fig. III-5 shows the influence of the order of titrant addition. When KOH was used as the first titrant, the subsequent curve of added HCl does not overlap with the KOH one (**Fig. III-5a**). Oppositely, when KOH was added after HCl running finished, the curve of KOH overlaps with HCl one accurately (**Fig. III-5b**). This effect can be attributed to the initial slow response of the glass electrode to the basic media, what leads to a gap in the corresponding potentiometric titration curves. Considering this effect, HCl was selected as initial titrant and subsequent KOH for back titration in this study, otherwise the titrations will require very long time to complete.

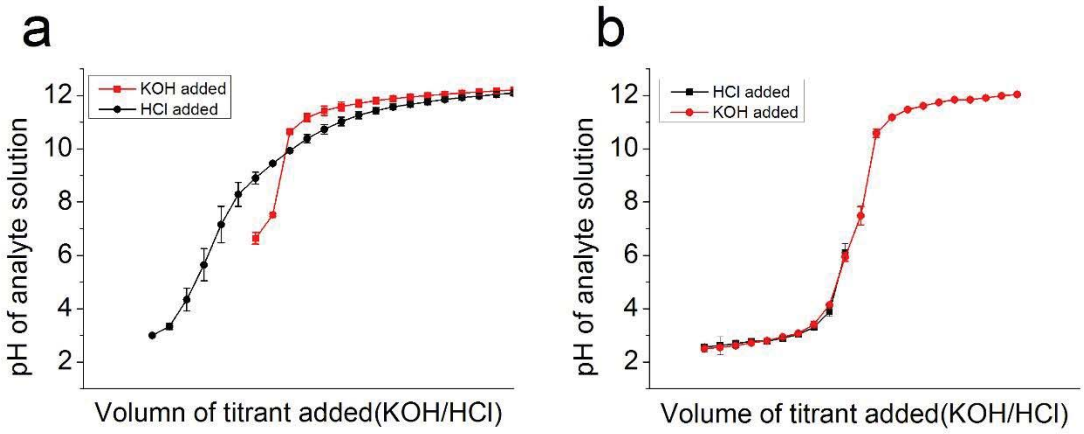

Figure III-5. Titration curve of the nutrient solution. KOH added firstly into the system (a), HCl added firstly into the system (b). Results shown are means ± SE (n=3).

Influence of the plants growing in the hydroponic nutrient solution

Measurements were performed under optimized conditions including samples of Hoagland nutrient solution after growing the plants (final nutrient solution) and the same solution before growing plants (initial solution). In the previous study has been reported that the root system can secrete an enormous range of compounds into the surrounding media[32] as for example organic acids, phytosiderophores, amino acids and sugars.

As shown in **Fig. III-6,** titration curve can interpret those changes by reflecting on the shape of the curve. In strongly acidic conditions, the hydrogen Se(IV) and Se(VI), $HSeO_3^-$ $HSeO_4^-$, is formed, respectively. Se species did not affect the signal in the initial nutrient solution neither in the final one **(Fig. III-6a, b)**. Thus, the titration curves graphs were quite similar for all the Se treatments**.**

The root exudates do affect the titration curve showing different shape in the final nutrient solution compared to the initial nutrient solution in absence of plants (**Fig. III-6c, d, e, f**).

The observed differences are attributed to the weak acid-base compounds that are exudated from the plant root system.

Only one inflexion point at pH 10.5 was observed in the curve of the initial nutrient solution **(Fig. III-6b)**. While there were two inflexion points in the curve corresponding to the final solution, one at a pH of 6.0, and the second at pH around 10.0 **(Fig. III-6a)**. The reaction is obviously happening in two distinct steps which means that the components in the final nutrient solution can exchange 2 protons from compounds of the root exudates. It has been pointed out [32] that carbon-based compounds are dominant in root exudates, which can often be separated into two classes, low-molecular weight compounds that include amino acids, organic acids, phenolic substances, etc. and high-molecular weight compounds like proteins. Such compounds can be responsible of the observed acid-base behavior.

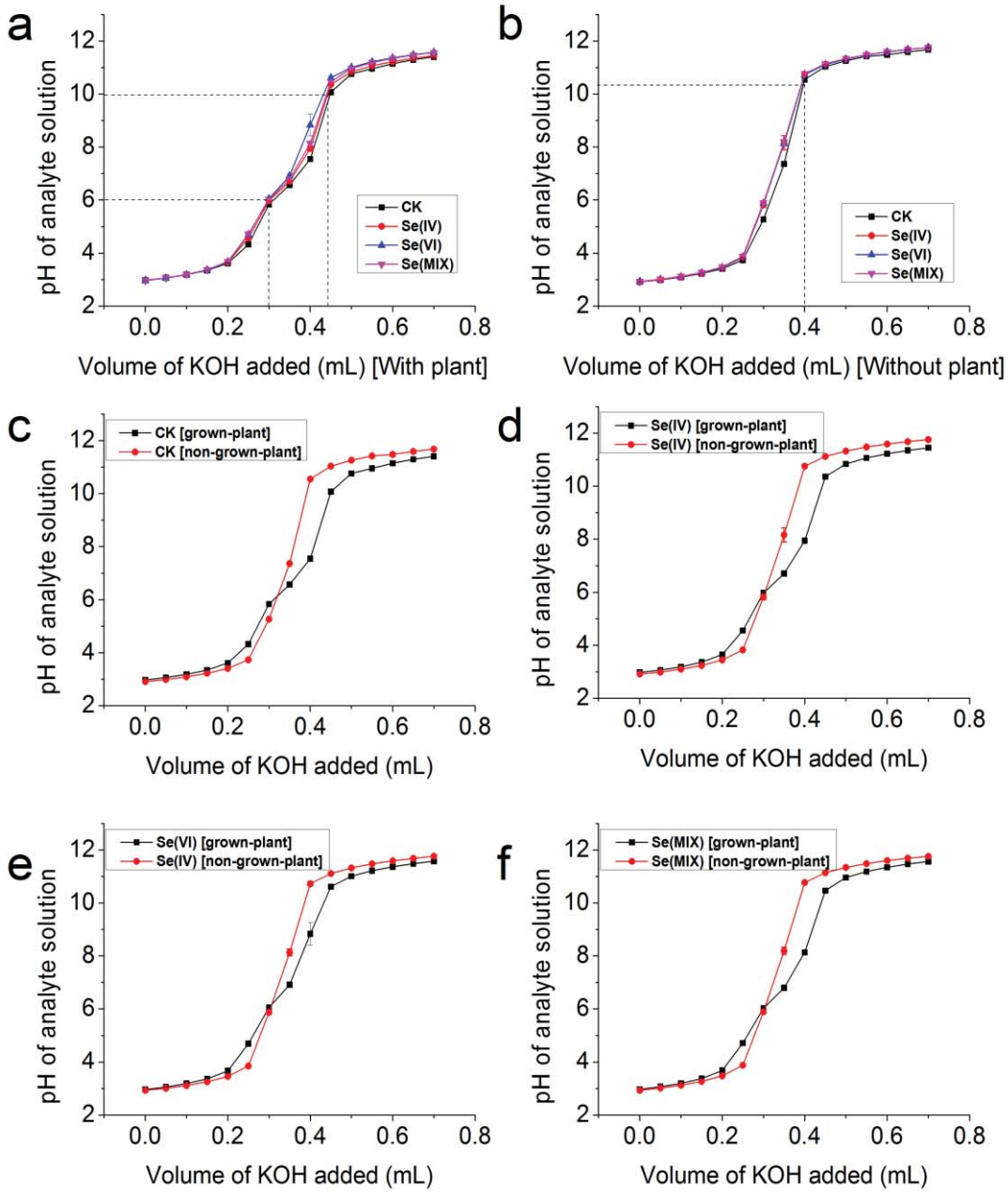

Figure III-6. Titration curves of the Hoagland nutrient solution with 0.05 M KOH as titrant added into the system. Overlapped titration curves of the final (a) and initial (b) Hoagland nutrient solutions; Individual titration curves of CK-Control (c), Se(IV)-Selenite (d), Se(VI)-Selenate (e), and Se(MIX)-Selenite: Selenate=1:1 (f). Results shown are means ± SE (n=3).

Xylem sap

The signal in xylem sap collected from Se enriched wheat plants were analyzed by titrator shown in **Fig. III-7**. Selenium species affected the curve shape and inflexion point in this case. All curves were with two inflexion points at different pH: In control samples, the inflexion points at pH 5.0, 7.39; In Se(IV), the equivalent points at pH 5.23, 8.01; In Se(VI), the inflexion points at pH 5.46, 7.11; In Se(MIX), the inflexion points at pH 5.42, 7.31. The curve corresponding to CK was steeper than those exposed to Se species. The curve of Se(MIX) was closer to the control one while a bit far away from the Se(IV) and Se(VI) curve. It could be the weak acids in Se(IV) and Se(VI) treatment sap were milder than those in Se(Mix) and control sap.

The pH corresponding to the inflexion point could be neutral acidic or basic, it depends on the salt produced by the reaction of base and acid. In present study, we found two inflexion points in titration curves, the first one was acidic around pH 5.0. The second was basic over pH 7.0. It indicates that there are diprotic acids released in xylem sap. In principle, malic acid is the dominating organic acid founded in maize sap [33]. Citrate and malate are the predominant organic founded in *T.aestivum* plants [6]. Typically, the conjugate base that is formed from the first dissociation of a polyprotic acid is still considered a weak acid itself and will undergo hydrolysis with water. Therefore, it produces a pH at the first inflexion point that is less than 7.0.

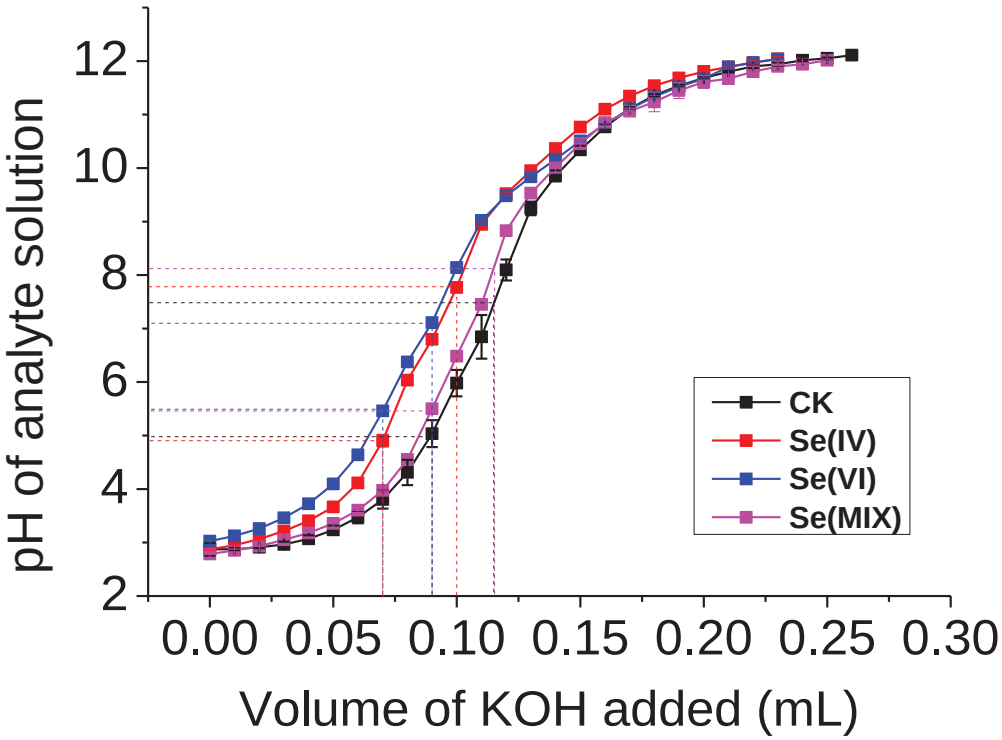

Figure III-7. Titration curve of xylem sap with 0.05 M KOH as titrant added into the system. Results shown are means ± SE (n=3).

Conclusions

Wheat plant alkalify the nutrients after uptake by root in hydroponics cultivation. Se species do not affect the pH in xylem sap. Selenate is the main species mobile from root to shoot via xylem transport with low species transformation micronutrients such as Cu, Zn, Mo decreased by Selenite indicates that Se(IV) plays more hazard role than Se(VI). The concentration of mineral elements in the xylem sap influences their accumulation in the shoot, thus, root to shoot translocation is a key process to control Se and nutrient accumulation in the plant aerial part.

A quick and low-cost method for testing the signal has been established. As a supplementary technique, titration method provides a possible research field with advantages of simple pretreatment and low-cost.

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Chapter IV

Influence of a plant biostimulant on the uptake, distribution and speciation of Se in Se-enriched wheat (*Triticum aestivum* L. cv. Pinzón)

Abstract

Selenium (Se) can induce stress in plants. Our main purpose is to assess the effect of a biostimulant, based on heteropolyoxometalates mixed with humic acids, on Se bio-fortified wheat plants. Secondly, to evaluate a possible modification of the Se species in the plant tissues and to investigate the response of phytohormones.

Wheat plants were grown hydroponically and exposed to either selenite (Se(IV)), selenate (Se(VI)) or a mixture of both species (Se(MIX) in the presence or absence of the biostimulant (foliar, FA; or root application, RA). Biomass, mineral nutrient concentration, phytohormones and Se speciation were investigated.

The biostimulant FA did not modify the plant biomass but RA significantly increased the root biomass in all treatments as well as the shoot biomass of plants exposed to Se(VI) and Se(MIX) even when both biostimulant modes of application caused a severe reduction of IAA levels in shoots. The biostimulant accelerated the translocation of Se from roots to shoots in the presence of Se(VI) and Se(MIX), and only had a noticeable influence on the Se speciation in roots, not much in shoots. X-ray absorption spectroscopy allowed to identify organic Se as the main Se species formed in the plant shoots being the influence of the plant biostimulant almost negligible on the Se speciation.

The biostimulant has a remarkable influence on both the uptake and accumulation of certain mineral nutrients and the plant metabolism by increasing the plant biomass under Se exposure. This indicates the potential of this biostimulant that also will prevent the possible Se-induced stress.

Key words: Se speciation, Plant biostimulant, Phytohormones, XANES, Selenite, Selenate.

Introduction

Selenium (Se) is an essential micronutrient for humans since it is an important constituent for proteins, where substitutes sulfur (S) atoms and acts as the active center of enzymatic processes [1,2]. Appropriate selenium intake can be beneficial for human health due to its anti-oxidant, anti-viral and anti-carcinogenic properties [2,3]. As it has been mentioned in **introduction section (chapter I)**, the bioavailability of Se is intimately related with its chemical form. In general, organic Se species are more bioavailable than inorganic ones, for which the bioavailability can reach values up to 80-90%, whereas only 20% of inorganic Se is able to reach to the circulatory system in human body [4,5]. As it has been shown in previous studies [6–8], plants are able to transform the Se inorganic species present in the soil into seleno-amino acids in tissues, which are the forms of selenium desired for animals and human intake. Therefore, the Se present in our diet comes indirectly from the reservoir in soils, mainly via plants and/or meat consumption. Thus, regions with low Se level in soils provide Se deficient diets. In order to overcome this problem, the elaboration of functional foods from edible plants through Se-enrichment procedures has been proposed as a solution [9]. In our study, wheat has been chosen as an ideal candidate due to its large consumption worldwide. Although the application of Se-containing fertilizers is becoming an increasingly common practice in many countries such as Finland, Norway and UK where Se levels in soil are especially low [5], there are still issues to be addressed regarding the observed toxicity of Se to the plant itself. Moderate to high doses of Se are necessary to reach appropriate Se content in edible plant parts and they can be a source of stress to the plant and hamper its normal development [10]. This is mainly due to the fact that Se substituted proteins do not accomplish any metabolic function for the plant [11]. Therefore, growth is reduced, lowering the yield, which would imply big economic losses for agriculture as well as a threat to the environment.

In this respect, the use of anti-stress agents, also called plant biostimulants, could help to overcome this drawback. These products are used to improve nutrient efficiency, abiotic stress tolerance and crop quality [12]. Despite this being an increasing field of research, only few previous works [13,14] have explored the possibility of applying a biostimulant to crops exposed to selenium fertilizers. In the study from Seciu [15], a biostimulant containing Se in its composition was applied directly and the alleviation effect of Se on water shortage was analyzed but not the amount of Se in cauliflower or cabbage. In our work, we have applied a plant biostimulant based on a complex of hybrid hetero-polyoxometalates (HPAs) of Keggin structure molecules mixed with humic acids, called "Phyto-Fitness" (BIO Fitos S.R.O., Czech Republic). Our aim is to assess the effect of this product in counteracting the toxicity of Se which hampers the normal development of wheat plants. Hence, this study will help to develop the most efficient procedure to biofortify crops with this essential element for humans. Special attention is paid to plant biomass, plant growth hormones and the effect of Se on micro and macronutrient concentration as well as to the identification of the Se species present in different parts of the plant to evaluate a possible modification on the Se speciation by the biostimulant.

Materials and Methods

Chemicals

$CuSO_4 \cdot 5H_2O$ (≥98%), Cellulose, $CaCl_2$ (95%), KNO_3 (99%), $Ca(NO_3)_2 \cdot 4H_2O$ (99%), FeNa-EDTA (99%), H_3BO_3 (99%), $(NH_4)_6Mo_7O_{24}$ (99%), KOH (85.5%), KH_2PO_4, MES (2-morpholinoethanesulfonic acid)(99.5%), $MgSO_4 \cdot 7H_2O$ (≥99%), $MnCl_2 \cdot 4H_2O$ (99%), $ZnSO_4 \cdot 7H_2O$ (98%), (VWR, Barcelona, Spain); HOAc, MeOH, 2-Propanol, (VWR, Barcelona, Spain); Na_2SeO_3 (AMRESCO, Barcelona, Spain); Na_2SeO_4 (FLUKA,

Barcelona, Spain); 3-Indoleacetic Acid (IAA) and Indole-3-Butyric Acid (IBA), IAA (IAA-d5 98 atom % D), Seleno-L-methionine, Seleno-L-cystine and Se-(Methyl) selenocysteine hydrochloride (Sigma-Aldrich, Madrid, Spain). All the reagents used were of analytical grade.

Phyto-fitness (BIO Fitos S.R.O., Czech Republic). Consist 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.

Plant material and Se treatments

Wheat was germinated and grown as describe in general **methodology section.** In order to evaluate the effect of the plant biostimulant, Phyto-fitness, three batches of plants were grown under the following conditions: No biostimulant (NB); foliar application (FA) of the biostimulant by spraying the product 100 times diluted on the leaves (one single foliar application); root application (RA) of the biostimulant through the nutrient solution, 1000 times diluted and renewed with the nutrient solution with a final concentration of 0.37 μM Mo, 1.7 μM B and 0.5 μM V being the rest of elements under the detection limit.

After two weeks of preculture in these conditions, plants were further exposed for two more weeks to different selenium treatments: Control (No Se); 10 μM sodium selenite, Na2SeO3 (“Se(IV)” treatment), 10 μM sodium selenate, Na2SeO4 (“Se(VI)”treatment); and a 1:1 v/v mixture of both reaching a concentrations of 10μM Se (“Se(MIX)” treatment) (**Fig. IV-1**).

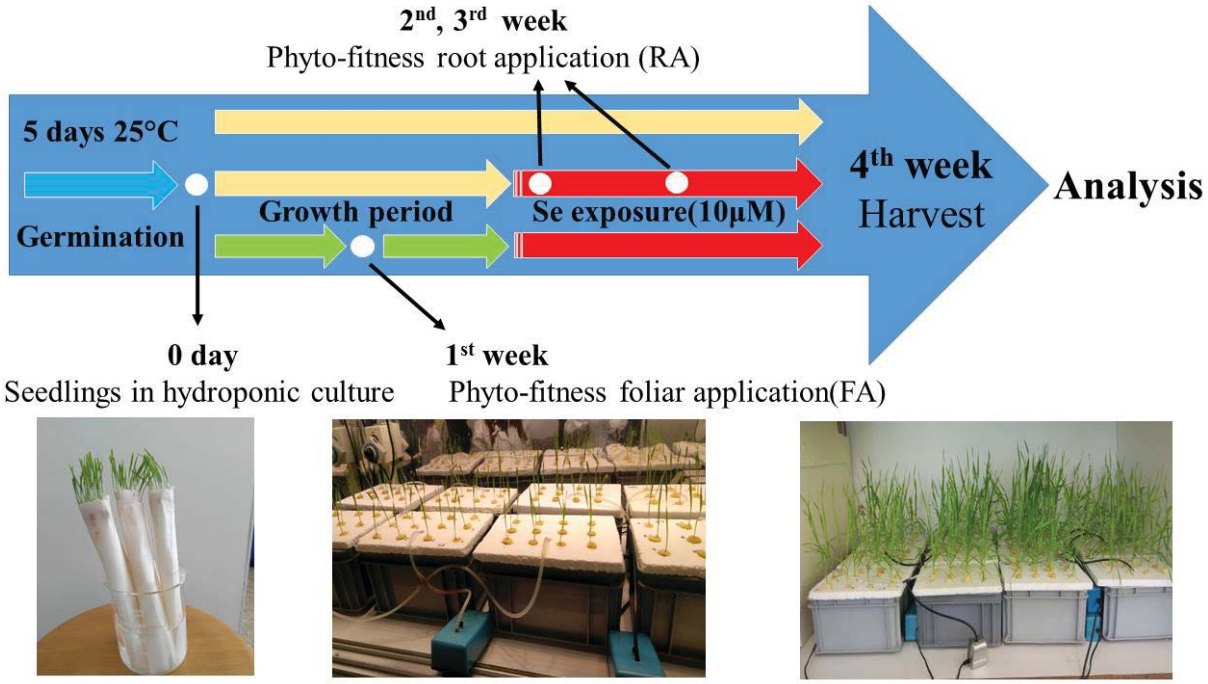

Figure IV-1 Schematic diagram showing the experimental design.

Sample Collection and Growth Parameters

After four weeks, plants were harvested; roots were desorbed with 10 mM $CaCl_2$ cold solution to remove Se from the root apoplast and leaves washed with deionized water to remove any residual sprayed plant biostimulant. Afterwards, plants were separated into roots and shoots, lyophilized and weighed to study changes in plant biomass.

Plant auxins quantification

The endogenous growth plant hormones, 3-Indoleacetic Acid (IAA) and Indole -3-Butyric Acid (IBA), were extracted and purified as modified by Llugany [17]. Briefly, 250 mg of fresh material was milled in an ice-cold mortar with 750 µL extraction solution constituted by MeOH: 2-Propanol: HOAc (20:79:1 by vol.). Then, the supernatant was collected after centrifugation at 1000 g for 5min at 4℃. These steps were repeated two

more times and pooled supernatants were lyophilized. Finally, samples were dissolved in 250 µL pure MeOH and filtered with a Spin-X centrifuge tube filter of 0.22 µm cellulose acetate (Costar, Corning Incorporated, New York, USA). Hormone quantification was done using a standard addition calibration curve spiking control plant samples with the 2 standard solutions of IAA and IBA ranging from 50 to 1000 ppb and extracting as described above. Deuterated IAA (IAA-d5 98 atom % D) at 30 ppb was used as internal standard in all the samples and standards and all of them were purchased from Sigma-Aldrich, Spain. Plant hormones were analyzed by LC-ESI-MS/MS system in multiple reaction monitoring mode (MRM) according to Segarra [18]. First hormones were separated using HPLC Agilent 1100 (Waldrom, Germany) on an Acquity UPLC BEH C18 2.1 x 100 mm ID, 1.7 µm column (Waters, USA) at 50°C at a constant flow rate of 0.8 ml min-1 and 10 µl injected volume. The elution gradient was carried out with a binary solvent system consisting of 0.1% of formic acid in methanol (solvent A) and 0.1% formic acid in milliQ H_2O (solvent B) with the following proportions (v/v) of solvent A (t (min), %A): (0, 2) (0.2, 2), (1.6, 100), (2, 100), (2.1, 2) and (3, 2).

MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, Ontario, Canada). All the analyses were performed using the Turbo Ionspray source in negative ion mode for IAA and the respective deuterated standard and positive ion mode for IBA.

Total Se and mineral nutrient analysis

Powdered plant samples (n=4) were predigested with HNO3:H_2O_2 (7:3, v/v) (VWR, Barcelona, Spain) overnight then digested in hot block (Charleston, South Carolina, US) at 110 ^oC for 2 h. Mineral nutrients were analyzed by ICP-MS (Perkin Elmer Optima 8300, Barcelona, Spain) and ICP-OES (Perkin Elmer Nexton 350D, Barcelona, Spain).

Blanks were included in each batch of samples for quality control. Selenium and the macronutrients phosphorus (P), sulfur (S), potassium (K), magnesium (Mg), calcium (Ca), and micronutrients molybdenum (Mo), zinc (Zn), copper (Cu), iron (Fe), manganese (Mn) and, boron (B), were measured, but only Se, S, Ca, Mo, Zn, Cu, Mn were selected to discuss in this study since the other elements showed no significant differences between treatments.

Synchrotron based X-ray Absorption Spectroscopy measurements

The element selectivity of X-ray absorption spectroscopy (XAS) provides a unique insight to determine the speciation of Se in biological samples with mild sample manipulation. Alternative speciation methods based on the hyphenation of liquid chromatography and mass spectroscopy, such as HPLC-ICP-MS, requires sample pretreatment, which could modify the Se species under study and alter the results. Hence, XAS provides a direct insight of the chemical species in plant tissues.

For the measurements, 12 replicates were ground, and the resulting powder was pressed into a pellet. X-ray absorption near edge structure (XANES) spectra were collected at Se K-edge using QEXAFS scanning mode and Si (311) monochromator crystals at CLAESS beamline of ALBA Synchrotron [19]. The spectra were collected on several spots (3-5) on each pellet to account for possible inhomogeneities when mixing the powders of replicates for each sample. All the measurements were performed at liquid nitrogen temperature to avoid radiation damage of the samples. Due to the low concentration of Se in the plant samples, the measurements were performed in fluorescence mode using the multi-element Si drift detector with XSpress3 electronics.

Additionally, sodium selenite, sodium selenate, seleno-L-methionine, seleno-L-cystine and Se-(Methyl) selenocysteine hydrochloride (Sigma-Aldrich, Madrid, Spain) were

measured as Se references since they are the species expected to be present in the plant. Aqueous solutions of the appropriate concentration to obtain an optimum absorption jump (100-200 mM) were measured for each reference compound. The solutions were loaded into an in-house designed liquid cell with a 3mm transmission path and Kapton windows [20]. The references were measured in transmission mode using gas ionization chambers filled with the appropriate amount of nitrogen, argon and krypton to absorb 20% (I0) and 80% (I1 and I2) of the beam. Data normalization and analysis using linear combination fitting was carried out with Athena program of the Demeter software package [21].

Statistics

To check the reproducibility of the results, the entire experiment was repeated twice in different seasons; spring and summer. The results given are means (n=4) with standard error (±SE). All the data were checked for normality using Kolmogorov- Smirnov test and data not normally distributed were log transformed in order 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 done with Statistica software version 6.0 (StatSoft Inc., St. Tulsa, OK, USA).

Results and discussion

Plant biomass

A significant increase of root dry weight has been observed when the biostimulant was applied to the solution (RA) regardless the absence or presence of any of the Se species supplied **(Fig. IV-2a)** while no root biomass increment was observed when the biostimulant was sprayed onto the leaves (FA treatment). On the other hand, shoot

biomass **(Fig. IV-2b)** was only significantly increased by RA treatment under Se(VI) and Se(MIX) exposures. FA and Se treatments had no significant influence on the plant dry weight.

Previous studies have proved that plant biostimulants have a positive influence on biomass by stimulating the rhizosphere [22] activity and enhancing the antioxidant potential of plants [12]. Other works have reported that biostimulants formulated with humic substances may increase root growth through auxin activity [23]. This is in accordance with our results where RA of the biostimulant, formulated with humic acids, had better efficiency than the FA at least on hydroponic short-term grown plants. Our biostimulant had no effect on the biomass of Se(IV) exposed plants neither on the shoot dry weight of control plants. Therefore, it can be concluded that Se species is a significant factor influencing the action of the biostimulant in this short-term study.

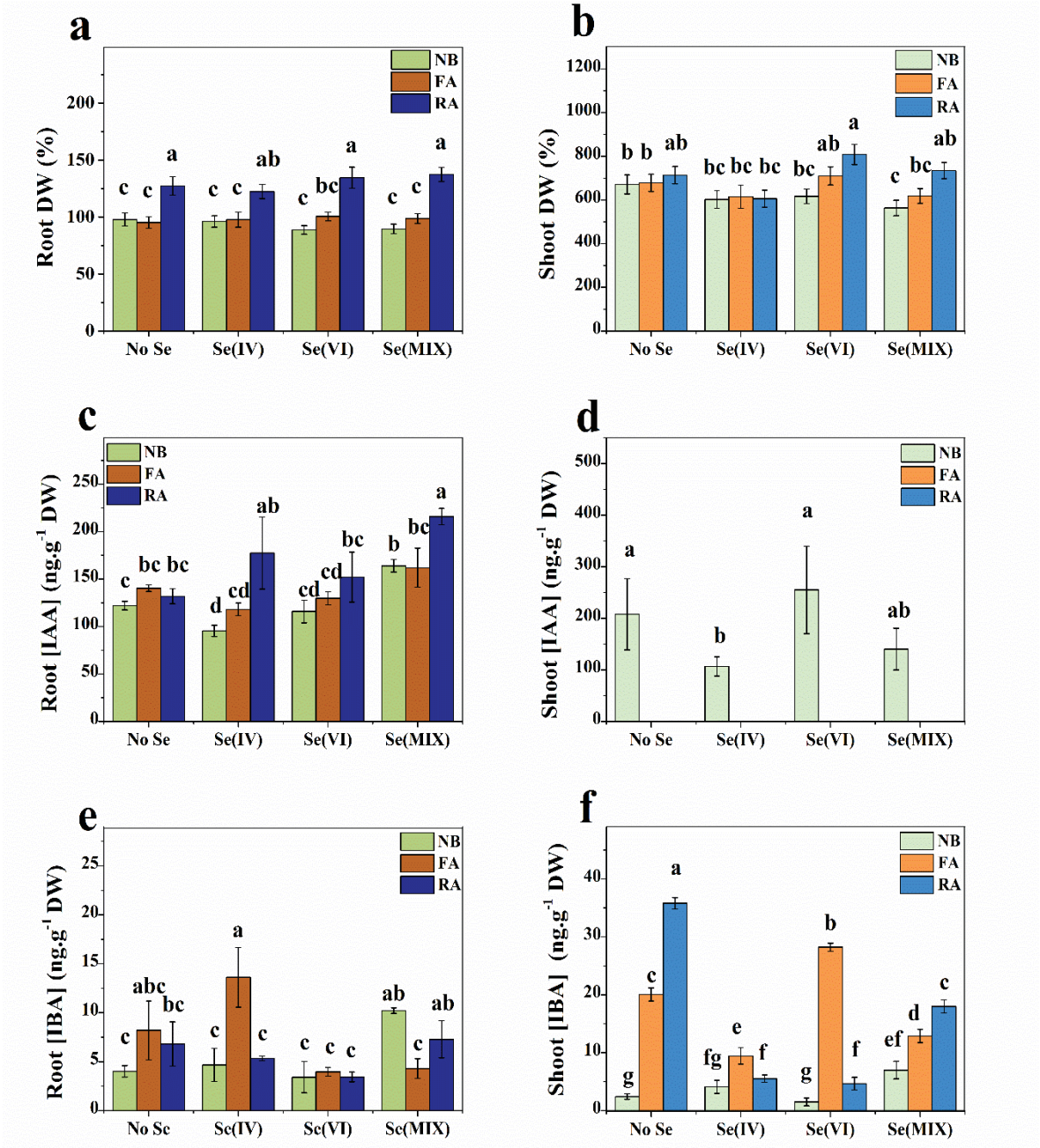

Figure IV-2 Dry weight and concentration of IAA and IBA in roots (a, c, e) and shoots (b, d, f), respectively of *T. aestivum* plants grown under different Se species (selenite, selenate and mixture of Se) at 10 µM and biostimulant application (No biostimulant (NB), Foliar (FA) and Root (RA)). Results shown are means ± SE (n=24), n=3, respectively. Different letters indicate statistically significant differences among groups (LSD, $P<0.05$).

Plant Auxins

Auxinic hormones are important regulators of plant growth and development because they play a critical role in cell division and cell expansion [24,25]. Auxin biosynthesis occurs in both aerial parts of the plant and in roots. In addition to biosynthesis of the main auxins via tryptophan converted to indole-3-pyruvic acid (IPyA) pathway, the pool of active auxin IAA can be modulated by inputs from additional storage forms and precursors, such as IAA conjugates and IBA in many plants [26,27].

In our study, we found that Se(IV) significantly reduced IAA levels in roots and shoots of wheat plants without any biostimulant treatment (NB) **(Fig. IV-2b, c)**. In roots, the FA treatment had no influence on IAA levels while RA treatment increased IAA concentration of plants exposed to Se(IV) and Se(MIX) above control ones (NB, No Se). Both FA and RA treatments caused a severe reduction of IAA in shoots to levels under the detection limit **(Fig. IV-2d).** The increment in root biomass observed in those previous studies is in accordance with our results where RA treatment had better efficiency than the FA, at least on hydroponic short-term grown plants enhancing IAA levels in roots. Surprisingly, in shoots the response was different **(Fig. IV-2c, d)**. It seems that the biostimulant may inhibit the basipetal transport of auxin from stem tip to root so the auxin detected in the root is presumably root apically synthetized IAA and/or auxin converted from storage IAA conjugates and from the precursor IBA. In *Triticium aestivum*, IBA may be stored in amino acid conjugate form [28] and seems to be transported by carriers different to those of IAA [29].

IBA levels of wheat plants without any biostimulant treatment (NB) were not affected by Se(IV) or Se(VI) treatments but Se(MIX) had a positive effect both in roots and shoots

(Fig. IV-2e, f). In roots, FA treatment increased IBA levels in Se(IV) exposed plants and avoided the increase detected in Se(MIX) treated plants whereas RA treatment had no effect on IBA root levels. In shoots, FA and RA treatments enhanced IBA concentration in all the treatments excepting for RA plants exposed to Se(IV).

It has been reported that IBA is difficult to be identified in samples from *Arabidopsis*, *Populus* and wheat [30], however we did not have this difficulty even though the low concentrations of IBA detected in relation to IAA **(Fig. IV-2e, f)** that are in accordance with other reported IBA values [31,32].

IAA and IBA levels in shoots change in parallel **(Fig. IV-2d, f)** with the exception of Se(IV) treatment; when IAA levels raised, IBA levels correspondingly decreased in the same group. The differential behavior of Se(IV) can be due to the fact that this Se species can alter auxin biosynthesis and transport as it is pointed out in rice seedlings by Malheiros[33], where an increase in the primary root length but a decrease in the number and length of lateral roots was observed. In our study, Se(IV) reduced IAA levels in both roots and shoots but no significant effects on plant biomass under any of the Se treatments (NB), although the length of the primary and lateral roots were not measured. In roots, this reduction is counteracted and even an increase is observed in comparison to control for biostimulant FA and RA applications, respectively. This is in concordance with the increase observed in root dry matter. Thus, our short-term study reveals that the biostimulant has a great influence on the plant metabolism, especially on the growth hormone IAA, promoting an increase in biomass of the root system regardless of the presence or absence of selenium in the medium.

Total selenium and sulfur concentration in plant tissue

Selenium uptake and distribution

Root Se concentration in the Se(IV) treated plants is higher than for the other Se formulations used while the highest concentration of Se in shoots is found in Se(VI) treatment (**Fig. IV-3a, b**). Selenium concentrations in roots and shoots can directly reflect the Se uptake and assimilation in the plant tissues under the influence of the different Se species applied and the foliar or root biostimulant application. In this study, as in previous ones [10,34,35], Se(IV) is rapidly assimilated in roots and has limited translocation to shoots. In contrast, Se(VI) is readily absorbed in roots and transferred to shoots while Se(MIX) has an average behavior.

The biostimulant applied to either leaves or roots seems to cause a decrease of Se concentration in root when the Se is applied in the form of Se(VI). Under the Se(MIX) treatment, Se concentration in NB root was significantly lower than FA group and higher than RA **(Fig. IV-3a)**. Figure **IV-3b** shows foliar application of the biostimulant caused different responses in shoot depending on the Se species supplied to the plants. Under Se(IV) treatment, shoot Se-concentration remain constant while under Se(VI) and Se(MIX) treatments the amount of Se in shoot decreased and increased, respectively.

Foliar application of the biostimulant is a good choice for Se-enrichments in the form of Se(MIX) because enhances Se accumulation in shoots. If we take into consideration both Se accumulation and biomass production, the best choice is to apply the biostimulant at the root level (RA) where wheat plants produce more biomass and maintain Se levels similar to those of plants without the presence of the biostimulant. Oppositely, foliar

application of this biostimulant should be avoided in the presence of Se(VI), which significantly decreases the Se shoot concentration without enhancing the biomass.

The Se translocation factors, $[Se]_{Shoot}/[Se]_{root}$, [Se]=total Se concentration) (**Fig**. **IV-3c**) for plants with RA biostimulant is higher than the translocation factors of NB or FA plants dosed to any Se species, especially exposed to Se(VI). In other words, Se absorbed by root was strongly translocated to the aerial parts under RA. This suggests that the biostimulant applied through the root (RA) promotes Se translocation from roots to shoots in Se(VI) plants but it has no effect on Se(IV) ones resulting in an intermediate response with Se(MIX) exposures.

Interactions between Selenium and sulfate

Sulfur as a crucial element in plant has many similar chemical properties to Se [36]. Se supplement could affect S level in plants. The pattern of S accumulation (**Fig. IV-3d, e**) was like that found for Se (Fig. 3a, b) under the same experimental conditions, especially in shoot. In roots, Se(IV) and Se(MIX) seem to cause a slight significant decrease of S levels (**Fig. IV-3d**) while in shoots, Se(VI) and Se(MIX) caused a noticeably significant increase in S levels (**Fig. IV-3e**), independently of the application or not of the biostimulant.

The high S level observed in shoots under Se(VI) exposure is in accordance with the work from Guerrero [10] . They found that sulfate uptake was not reduced in wheat plants by Se(VI) competition, but S distribution was strongly affected by Se exposure. Moreover, a study conducted by Bell [37] on the antagonism between Se(VI) and sulfate concluded that non-accumulators discriminate against Se(VI) uptake relative to sulfate. They

observed that non-accumulator plants had increased shoot S concentration by increasing Se(VI) in solution, but only when shoot Se was above 20 $\mu g.g^{-1}$ DW. In our study, wheat plants that presented these high S levels in shoot had above 200 $\mu g.g^{-1}$ Se DW, while plants with no change in shoot S levels had values near 25 $\mu g.g^{-1}$ Se (**Fig. IV-3b**). Selenate-induced stimulation of S uptake is the result of incipient Se toxicity [10,37,38] and the high S levels found in shoot of Se(VI) and Se(MIX) group of plants is due to the stimulation of sulfate transporters expression by Se(VI), resulting in transcriptional changes resembling those induced by S starvation, which facilitates sulfate uptake and S assimilation in wheat plants as it was proposed by Boldrin [39], White [40], and Schiavon [41]. On the other hand, it has been reported that S in shoots is not always corresponded with the Sultr2;1 transcript levels, because other Sultr genes (Sultr2;2, Sultr3;5) also contribute to S translocation hindering thus the well-known sulfate-selenate transport competition [42,43].

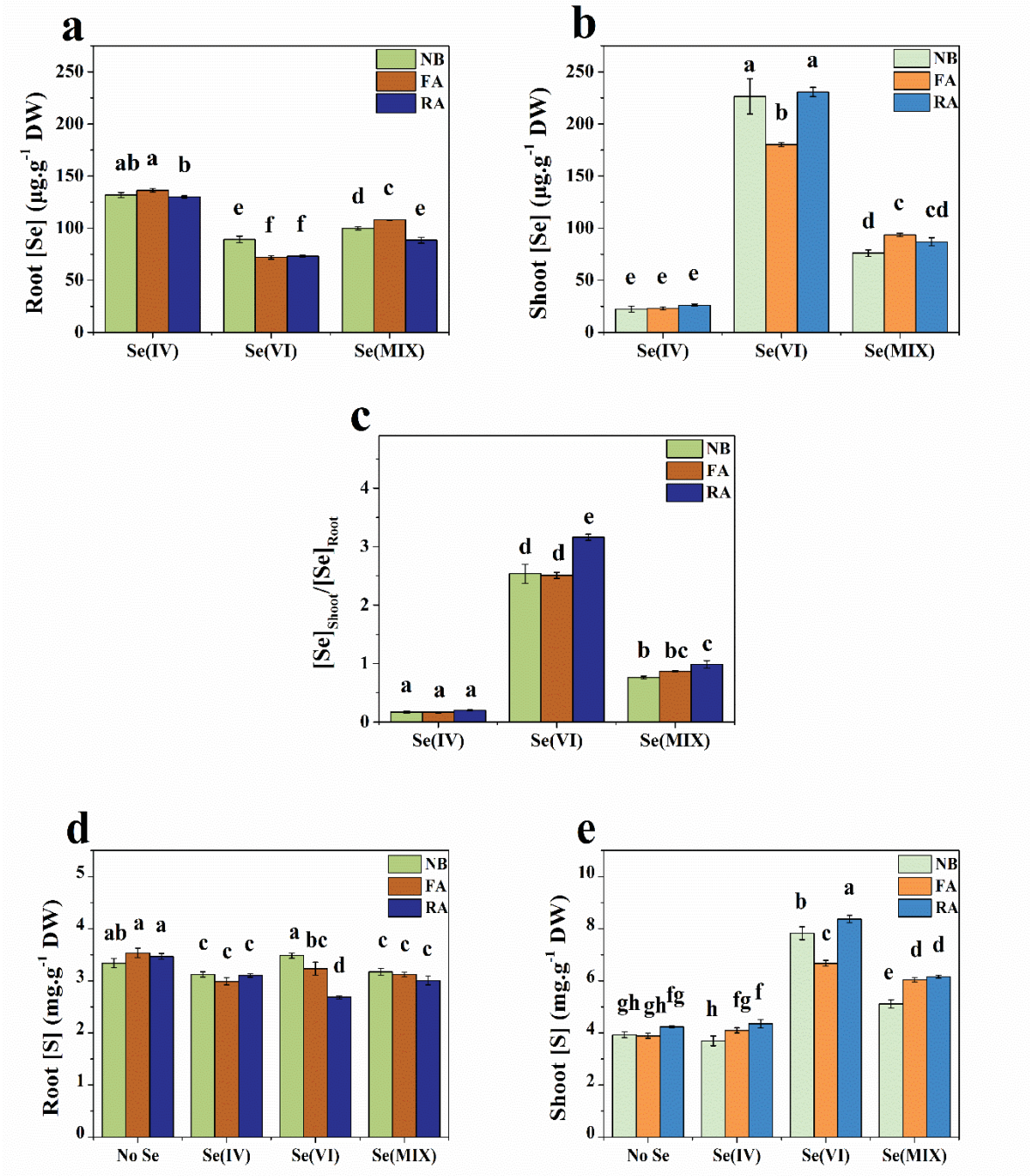

Figure IV-3 Concentration of Se and S in roots (a, c) and shoots (d, e), and Se translocation factors (c) of *T. aestivum* plants grown under different Se species at 10µM and biostimulant applications (No biostimulant (NB), Foliar (FA) and Root (RA)). Results shown are means ± SE (n=4). Different letters indicate statistically significant differences among groups (LSD, $P<0.05$).

Effects on Micro- and Macronutrients

Ca is an important element to evaluate due to its role in the stability and function of the plant membranes and cell wall structure [22,44]. The application of the biostimulant through the root system (RT) caused a remarkable increase in root and shoot Ca levels regardless of the Se treatment. In contrast, biostimulant foliarly applied (FA) caused a slight increase in root Ca concentration under Se(IV) treatment and in shoot under Se(VI) and Se(MIX) exposed plants (**Fig. IV-4a, b**).

Mo is another essential micronutrient for plants since it participates in the nitrogen fixation [45,46]. A significant increase of Mo levels in roots and shoots has been observed when the biostimulant was applied to the solution (RA) regardless the absence or presence of any of the Se species supplied (**Fig. IV-4c, d**). It is due to Mo presenting micromolar amounts in RA. Mo has a wide variation between the critical deficiency and toxicity concentrations which may differ by a factor of up to 104 (0.1 – 1000 μg Mo g-1 DW) [47] and our results are within this range.

The increment of both Ca and Mo could be responsible of the enhanced root DW observed when the biostimulant was applied to the nutrient solution (RA). The beneficial effects of the RA treatment should not be ascribed only to Mo but to the cooperative/synergistic effect of the different components of the plant biostimulant which provides the aforementioned benefits for the plant. Actually, the isolation and study of single components present in a biostimulant can produce unreliable results because the effect on plants are often due to the combination and synergistic action of different compounds [12].

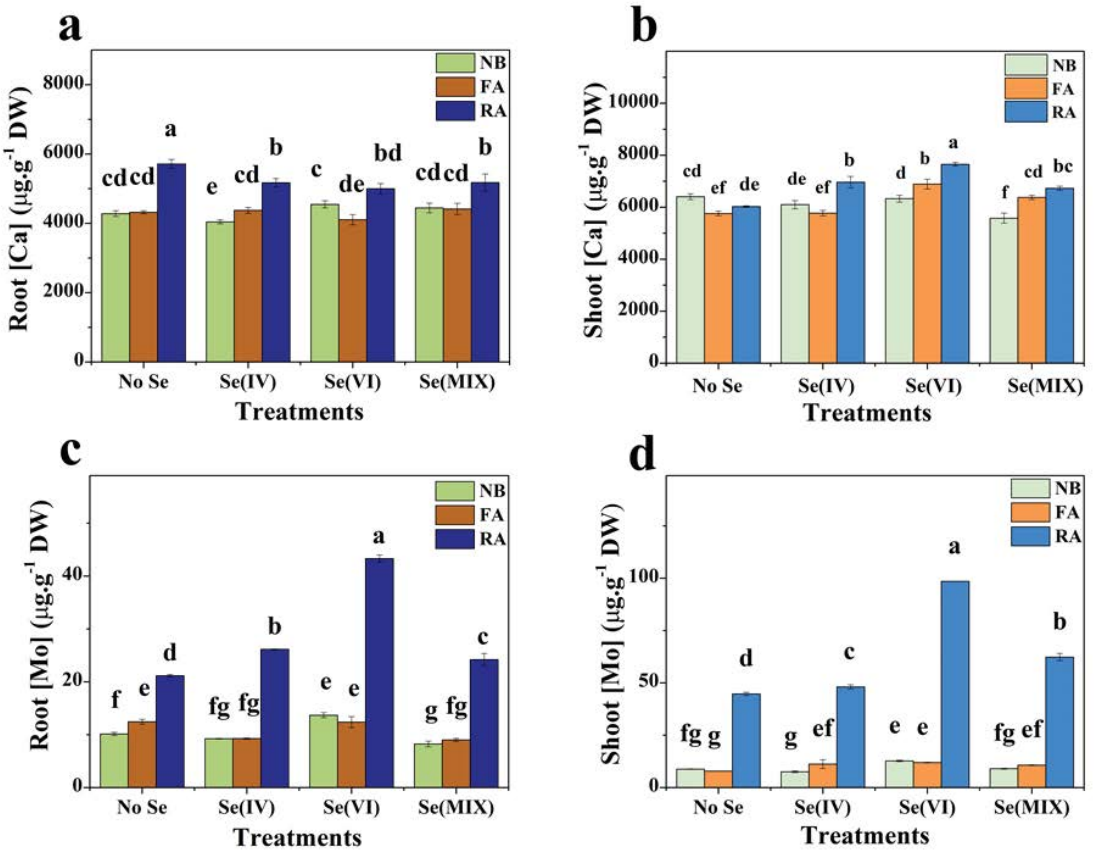

Figure IV-4. Concentration of Ca and Mo in roots (a, c) and shoots (b, d) of *T. aestivum* plants grown under different Se species (selenite, selenate and mixture of Se) at 10 µM and biostimulant applications (No biostimulant (NB), Foliar (FA) and Root (RA)). Results shown are means ± SE (n = 4). Different letters indicate statistically significant differences among groups (LSD, $P<0.05$).

Zn, Cu and Mn are also essential micronutrients for plant growth, which contribute as cofactors to structure and/or catalytic activity of diverse enzymes, as for example, SOD (Superoxide Dismutases), which play a key role on ROS (Reactive Oxygen Species) scavenging [48].

The application of the biostimulant through the root system (RA) caused a significant reduction in the root levels of Zn, Cu and Mn regardless of the Se treatment (**Fig. IV-5a, c, e**). RA treatment also decreased the shoot concentration of these three elements but

only when there was no Se supply in the nutrient solution or when Se was applied in the form of Se(VI) (**Fig. IV-5b, d, f**). Under the mixture treatment the concentration of Zn, Cu and Mn had intermediate values between Se(IV) and Se(VI) or even higher than those of Se(IV) treatment.

FA treatment (**Fig. IV-5**) only had a positive significant effect on root Zn accumulation without Se supply and under Se(IV) treatment. Cu concentration in root was significantly enhanced by the FA of the biostimulant in all the treatments except for Se(IV). In shoot, Zn and Cu levels had no improvement respect the control values. Finally, Mn in root increased with the foliar application under Se(VI) and Se(MIX) exposure.

The biostimulant used in this study contains humic acids and a mixture of HPAs. Humic acids exhibit a large sorption capacity to cationic substances, hence RA treatment can play a key role in controlling bioavailability, and mobility of metals such as Zn, Cu and Mn. Soluble forms of related metal complexes with humic acids complexes probably enable a slow and gradual release of these metals for plant uptake. Despite the root Zn, Cu and Mn low levels under RA treatment, their translocation to shoots was not so drastically influenced by RA treatment. Nevertheless, Zn, Cu, and Mn concentrations in both roots and shoots are above the critical deficiency concentrations.

On the other side FA treatment may stimulate the rate of transpiration of the leaves and thus increases the photosynthetic rate, so that the water absorption also increases with the consequent increase in the concentration of Zn, Cu and Mn in the root.

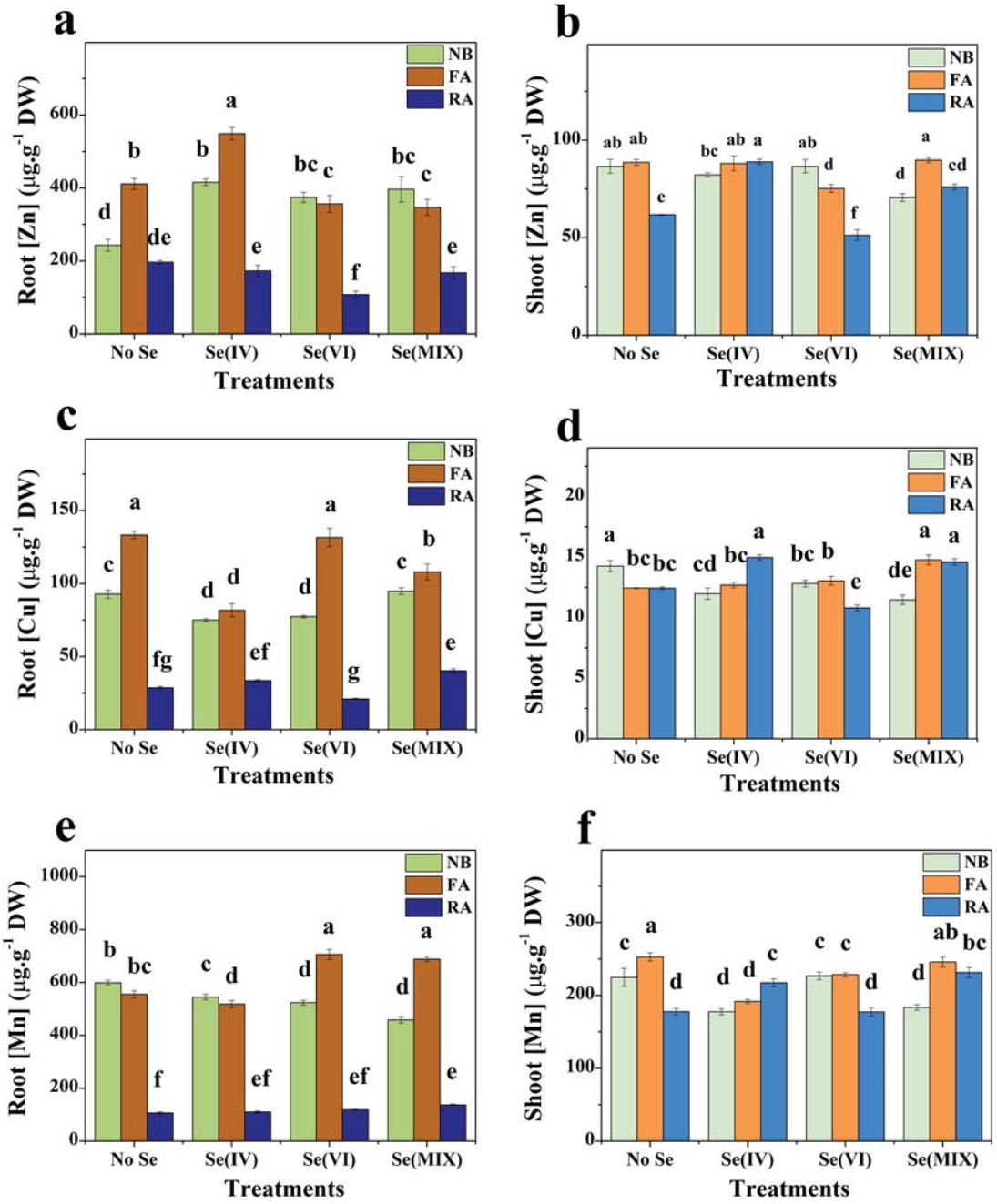

Figure IV-5 Concentration of Zn, Cu, Mn, in roots (a, c, e,) and shoots (b, d, f,) of *T. aestivum* plants grown under different Se species (selenite, selenate and mixture of Se) at 10 µM and biostimulant applications (No biostimulant (NB), Foliar (FA) and Root (RA)). Results shown are means ± SE (n= 4). Different letters indicate statistically significant differences among groups (LSD, $P<0.05$).

Selenium speciation by X-ray absorption spectroscopy

As mentioned above, plants are able to uptake and transform inorganic Se to seleno-amino acids through their metabolic pathway [6–8]. However, there are several parameters influencing the speciation of the Se accumulated in the plant. These are, among others, the culture conditions (hydroponics versus soil), concentration and type of Se applied (selenite versus selenate), the Se application methodology (foliar versus root) and the employment or not of plant biostimulant.

Selenium speciation in root

XANES is sensitive to the electronic configuration of the absorber atom and to the spatial arrangement of atoms around it [49]. Indeed, the Se reference compounds have characteristic features that allow their unequivocal identification. As shown in **Figure IV-6**, the spectra corresponding to the inorganic Se compounds dosed to the plants, *Ref selenite* and *Ref Selenate* showed characteristic prominent white lines (intense feature after the absorption edge) having their maxima at 12664 eV and 12667 eV, respectively. In addition, the position of the absorption edge for Se(VI) (E_0=12666 eV) was shifted 3 eV respect to *Ref selenite* (E_0=12663 eV). On the other hand, the white lines of the organic references measured are not as pronounce as the inorganic ones, and the absorption edges appear at lower energies: SeCyst (E_0=12659 eV), SeMet (E_0=12660 eV), MeSeCys (E_0=12660 eV). The organic references containing C-Se-C bond (SeMet, MeSeCys) have similar spectral shape characterized by a narrow white line having its maxima at 12661eV. Whereas the reference containing C-Se-Se-C bond (SeCyst) has a white line with maxima at slightly lower energy than C-Se-C bond, 12660eV. Likewise, E_0, in similar compounds is characteristic of the electronic structure of the Se in the system. As shown in **Figure 6**, E_0 for C-Se-C was higher than for C-Se-Se-C, 12660 and 12659 eV, respectively, which

indicates that there are less electrons closer to selenium in C-Se-C bond than in the C-Se-Se-C bond.

The comparison of these reference spectra with those collected on the samples allows us to perform a fingerprint analysis to obtain qualitative information regarding the species of Se in the different parts of the plant.

The plants grown without biostimulant (NB) are almost not affected by the Se-treatment used (**Fig. IV-6**). All the spectra have similar profile which resembles the one of the organic references but with a much wider white line. The spectra of Se(MIX) differs from the other two on the pronounced feature at 12667eV which can be attributed to a contribution coming from some *selenate* present in the NB-Se(MIX) root. On the other hand, when applying the plant biostimulant, FA and RA cases, the white line gets slightly narrower and its maximum was slightly shifted towards higher energies. In addition, the feature attributed to *selenate* was much larger than in the NB case for all Se feedings, and it grows as expenses of the white line intensity, being larger for the Se(IV) and lower for Se(VI). The white line of Se(MIX) is more similar to Se(VI) and the selenate-related feature is as intense as the one for Se(IV). These differences indicate that the plant biostimulant affects the chemical form of Se present in root, i.e., it affects the mechanism of Se assimilation. These spectral changes also suggest that the plant biostimulant induces oxidation of the Se(IV) during its uptake and assimilation in root. One possibility is that polyoxometalates as a constituent in the plant biostimulant used have a redox potential [50,51], which affects the transformation of selenite to selenate. However, the plant biostimulant slightly enhances the accumulation of Se(VI) in root in the presence of Se(VI), because the conversion of Se(VI) to Se(IV) by APR (ATP sulfurylase) occurs in the first stage of the Se assimilation pathway, mainly inside chloroplasts [52]. Then, Se(IV)

is transformed into organic forms. As expected, the spectral profile for Se(MIX) treated with biostimulant is a mixture of those of Se(IV) and Se(VI).

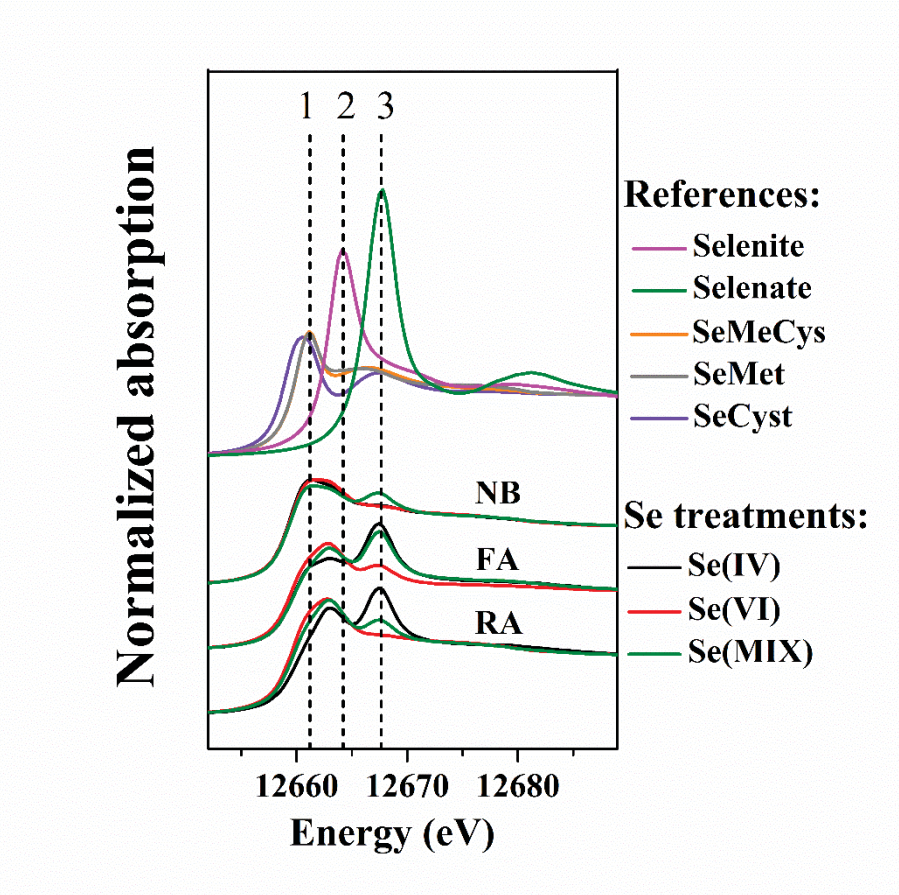

Figure IV-6. Normalized Se K-edge XANES spectra of Se references and root of the wheat plants grown under different Se treatments and biostimulant application. (No biostimulant (NB), Foliar (FA) and Root (RA)). The spectra have been shifted for shake of comparison.

Selenium speciation in shoots

Although the plant biostimulant application influences the Se speciation in root under the same Se treatment, the Se speciation in shoot was only affected by the different Se treatments used and slightly affected by the application of the plant biostimulant (**Fig.**

IV-7). Indeed, the XANES spectra for each Se treatment almost completely overlap (**Fig. IV-7a**). Se(IV) enriched shoot showed a broad white-line having its maxima at 12661eV that can be attributed to the presence of a C-Se-C bond. Se(VI) and Se(MIX) enriched shoot have the maximum of the white line at 12667 eV and a pronounced shoulder at lower energy, 12661eV, which suggest that *selenate* and C-Se-C bond are the predominant species.

In order to extract more quantitative information, we have performed a linear combination fitting (LCF) analysis of the XANES using the Se references mentioned above. The results can be used to estimate the ratio among the different Se species. The best fit and the percentage of the Se species found are shown in **Fig. IV-7b** and **Table IV-1**, respectively. In the shoot treated with Se(IV), organic Se species (C-Se-C and C-Se-Se-C) are found to be the main component, around 80-90% which suggests that organic Se are the primary species translocated from root to shoot with a low diffusion rate. Previous studies also support these results [7,53]. Selenium is chemically similar to S; hence, it is metabolized via S assimilation pathway inside the plant [11]. Indeed, Se(VI) is taken up actively by the sulfate transporter [52,54], whereas for Se(IV), it has been proposed that it can be taken up by the root through passive diffusion or by active absorption via phosphorus and silicon transporters [35,55,56]. Unlike Se(VI), the mechanism for Se(IV) is still not fully understood. In addition, Se(IV) is chemically closer to reduction into seleno-amino acid (protein retention mechanism) than Se(VI).

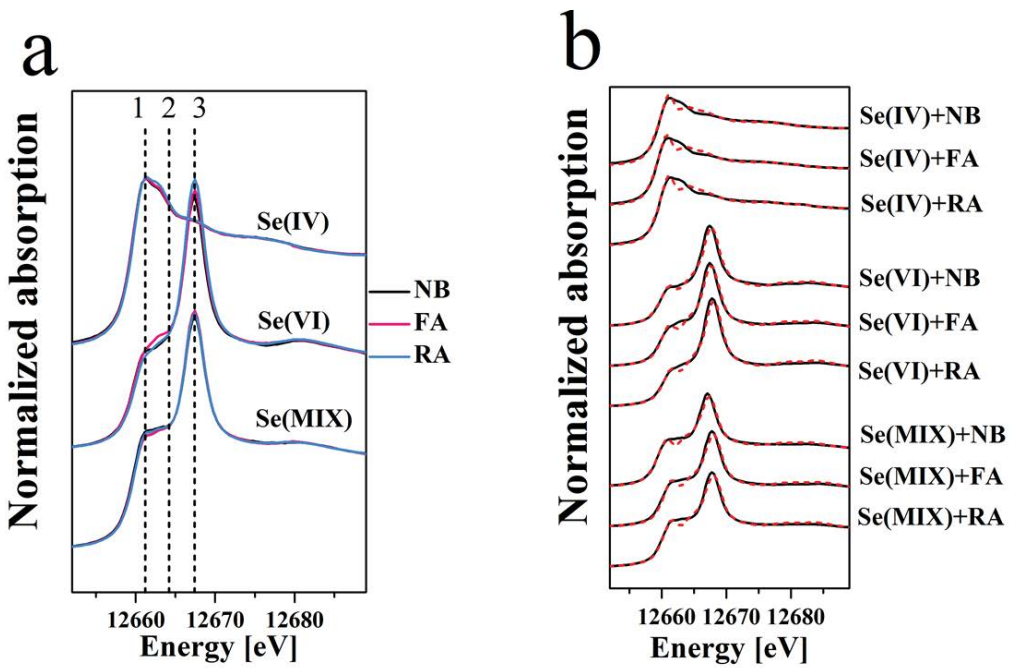

Figure IV-7 Normalized Se K-edge XANES spectra (a) of shoots of the wheat plants grown under different Se treatments and biostimulant application. (No biostimulant (NB), Foliar (FA) and Root (RA)). The spectra have been shifted for shake of comparison. Linear combination (b) fitting results (dotted lines) of shoots (solid lines) corresponding to (a), which obtained from Athena using reference compounds. Estimated percentages of reference compounds are shown in table 1.

Moreover, the assignment of specific Se compounds can be seen in **Table IV-1:** C-Se-C species: Se(IV)+RA (89.1%) > Se(IV)+FA (82.5%) > Se(IV)+NB (79%); which occurs as expenses of the concentration of C-Se-Se-C species: Se(IV)+RA (2.3%) < Se(IV)+FA (8.7%) < Se(IV)+NB (12.5%); inorganic Se was ~ 8.5% of *selenite*. Under Se(VI) and Se(MIX) treatment, there were ~ 10% of selenite, ~ 40-50% of *selenate* and ~ 35-57% of organic Se (C-Se-C), respectively. The lower amount of organic Se and higher amount of inorganic Se, especially Se(VI), are in agreement with a high mobility of selenate through the wheat xylem. As we mentioned above, ~ 10% C-Se-Se-C refer to SeCyst, formed from two SeCys molecules joined, which is more stable than SeCys, which was

only found in the NB+Se(VI) treatment. SeCys as the first organic form being non-specifically incorporated into proteins, leading to toxicity. While Se(VI)+FA and Se(VI)+RA accelerate SeCys converted to C-Se-C (SeMet, MeSeCys) with less toxicity. Despite the small differences found, this suggests that the biostimulant potentially enhances Se stress tolerance by reducing the accumulation and accelerating the conversion of SeCys under Se(VI) treatment. Nevertheless, further systematic studies addressed to the effect of biostimulants on Se speciation by using specific approaches are necessary to confirm these findings.

Table IV-1. Results from linear combination fitting analysis of Se K-edge X-ray absorption spectra collected on wheat shoot. The weight of each component is expressed as % of the total. The value in parenthesis is the uncertainty calculated as the standard deviation. Se(IV) and Se(VI) refer to selenite and selenate contributions. C-Se-C refers to components with a C-Se-C bond structure (e.g. SeMet and MeSeCys), and C-Se-Se-C to C-Se-Se-C bond structure (e.g. SeCyst). NB (no biostimulant application), FA (foliar application of the biostimulant), RA (root application of the biostimulant). See text for details.

Treatments	R-factor [a]	reduced χ^2	*Se(IV)*	*Se(VI)*	*C-Se-C*	*C-Se-Se-C*
Se(IV)+NB	0.0118	0.0032	8.5 (1.1)	-	79 (2.4)	12.5 (1.2)
Se(IV)+FA	0.0119	0.0031	8.4 (4.8)	-	82.9 (1.3)	8.7 (0.9)
Se(IV)+RA	0.0125	0.0037	8.6 (2.3)	-	89.1 (1.5)	2.3 (0.9)
Se(VI)+NB	0.0063	0.0027	11.3 (1.0)	40.8 (0.6)	35.4 (3.5)	12.4 (3.3)
Se(VI)+FA	0.0053	0.0023	10 (0.9)	42.7 (6.5)	47.3 (0.6)	-
Se(VI)+RA	0.0056	0.0026	10.1 (2.8)	47.2 (1.0)	42.7 (0.6)	-
Se(MIX)+NB	0.0055	0.0021	8.7 (1.4)	33.6 (0.6)	57.7 (1.0)	-
Se(MIX)+FA	0.0061	0.0024	9.5 (0.1)	35.3 (1.6)	55.2 (1.0)	-
Se(MIX)+RA	0.0057	0.0019	9.5 (0.9)	34.5 (2.7)	56 (0.9)	-

[a] R-factor is a measure of the mean square sum of the misfit at each data point which gives an indication of the goodness of fit. It is defined as: R-factor $= \frac{\sum(data-fit)^2}{\sum data^2}$

Conclusion

Our short-term study reveals that the biostimulant has a remarkable influence on the absorption and accumulation of certain nutrients, favoring the translocation of Se from roots to shoots. The biostimulant RA also influences the metabolism of the plant, especially the growth hormone IAA, that together with an increase in Mo may promote the increase in dry matter observed in the root system regardless of the presence or absence of Se in the medium.

Besides, the biostimulant affects mainly the Se speciation in roots while the Se speciation in shoots is essentially determined by the different Se treatments, being the influence of the biostimulant very mild. It seems that from roots to shoots translocation has balanced those varieties happened in roots. To some extent, the biostimulant can help plants to grow under Se exposure. The fact of overcoming or diminishing the toxicity induced by Se application has a great importance in agriculture and in biofortification programs since the productivity of crops would not be affected and the necessary dietary amount of organic Se forms in edible parts will reach an adequate range.

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Chapter V

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

Abstract

As already described in previous chapter I **Introduction**. An appropriate selenium intake can be beneficial for human health due to its antioxidant, antiviral and anti-carcinogenic properties. Although crop enrichment with selenium-containing fertilizers to obtain Se-biofortified food in Se-deficient regions is becoming an increasingly common practice, there are still issues to be addressed regarding the observed Se-induced toxicity to the plant itself. 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, Fyto-Fitness (by BIO Fitos S.R.O), to counteract the Se-induced stress in wheat plants was assessed. The co-application of different Se-biofortification treatments and the plant biostimulant at different growth stages (tillering or heading stage) was investigated in wheat plants until harvesting the mature grains. The use of micro focused X-ray spectroscopy techniques allows us to confirm that organic Se are the main Se species found in wheat grain whereas Se application stage slightly affects the proportion of organic Se. Our study proves that the biostimulant had a key role in enhancement both the amount of grains produced per spike and their biomass (DW) 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 selenium supplementation.

Key word: Se speciation, XRF, XAS, Wheat, Plant biostimulants

Introduction

As has been mentioned in general introduction chapter, the importance of selenium (Se) for human health has been widely confirmed in several human nutrient studies [1–4]. Se substitute 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 many chronic degenerative diseases [5–7]. To overcome this issue, Se supplementation has been extensively used (e.g. to control Keshan disease in China) [8,9]. 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 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[2]. Since 1984, soil fertilization with Se has been applied in Finland to increase Se concentration of food in regions with Se-deficient soils [10]. However, the presence of high concentration of Se produces stress to the plant and may hamper its normal development [11]. In order to overcome this issue, genetic engineering has been proposed as a strategy to enhance Se accumulation, volatilization and/or tolerance [12]. However, this approach has serious potential risks since it might promote the presence of new allergens in food [13], and it may promote the accumulation of other undesired heavy metals. Moreover, the elaborate procedures and challenges based on genetic engineering also need to be considered.

Alternatively, we propose to use a plant biostimulant based on hybrid hetero-polyoxometalates of Keggin structure mixed with humic acid (Fyto Fitness, BIO Fitos,

S.R.O., Czech Republic) as anti-stressor to alleviate the Se-induced toxicity in the plant. Despite that the application of anti-stressors is an increasing field of research in agriculture [14], only few previous works have explored the possibility of applying a biostimulant to crops exposed to selenium fertilizers. Peng [15] reported that fulvic acid as a biostimulant has beneficial effects and antagonists on the toxicity of selenite. In the study from Seciu [16], a biostimulant containing Se in its composition was applied directly to cauliflower or cabbage to evaluate the alleviation effect of Se on water shortage. 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.

Our main goal is to study the effect that the biostimulant has in counteracting the Se-induced toxicity which hampers the normal development of wheat plants. Hence, with the aim of maintaining the production yield, minimizing the plant toxicity and optimizing the total dosage of selenium supplementation, we applied different Se treatments (selenite, selanate 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 in order to study Se accumulation. In order to get information about the spatial distribution of Se and other relevant elements either for the plant metabolism (e.g. Se, Ca, Zn) or for human nutrition, μXRF measurements were performed. In addition, since the chemical state of Se is crucial to assess the biofortification procedure, μXANES measurements were collected at the most representative regions of the grain to get information about the Se speciation. This has allowed us to assess the possible modifications induced by the application of the plant biostimulant on the Se distribution and speciation on the wheat grain.

Methodology

Culture conditions and treatments

Wheat was germinated and grown as describe in the general methodology section.

In order to evaluate the effect of the plant biostimulant on the Se uptake and accumulation in the plant, two batches of plants were grown in the presence (FA-foliar application) or not (NB-No biostimulant) of the biostimulant. The foliar application of the biostimulant was done by spraying the product 100 times diluted on the leaves. Moreover, the plants were exposed to different Se treatments: no selenium (0 µM); selenite (Na_2SeO_3, AMRESCO, America), selenate (Na_2SeO_4, FLUKA, Spain) and a 1:1 v/v mixture of both substances at concentrations of 10µM Se.

In order to minimize both plant Se-induced toxicity and the economic cost of Se supplementation, one of the batches of plants was treated, as above mentioned, from the tillering stage and the other from the head emergence stage (heading stage) and both maintained until the grain became mature. Afterwards, plants and grains were harvested and kept until further analysis. See the schematic in **Fig V-S1**.

Total Se analysis

Powdered plant samples (n=4) were predigested overnight with HNO_3:H_2O_2 (7:3, v/v) (VWR, Barcelona, Spain) and then digested in hot block (Charleston, South Carolina, US) at 110C° for 2h. Mineral nutrient concentrations were analyzed by ICP-MS (Perkin Elmer Optima 8300, Barcelona, Spain) and ICP-OES (Perkin Elmer Nexton 350D, Barcelona, Spain). Blanks were included in each batch of samples for quality control.

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., St. Tulsa, OK, USA).

Samples preparation and Synchrotron based X-ray Absorption Spectroscopic measurements.

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

For the measurements, the specimens were mounted on the top of carbon tape disk which was stuck on to a sapphire disk which was then glued onto the Al holder for the liquid nitrogen cryostat using super-glue. XAS and XRF mapping measurements on the grain sections were performed at I18 beamline [17] of Diamond Light Source using a 4-element Si Drift fluorescence detector. The distribution of Se, Zn, Cu, Fe, K, Mn, Ca in grain were obtained from the XRF maps collected using an excitation energy of 12677 eV.

Results and Discussion

Grain biomass

Biomass parameters, such as the average dry weight (DW) of single spikes (**Fig. V-1a, b**) and of grains per spike (**Fig. V-1c, d**), and the number of grains per spike (**Fig. V-1e, f**) were monitored 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 (**Fig. V-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 (**Fig. V-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 head emergence. This is in agreement with the results found by Longchamp[18] 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[19] 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 (**Fig. V-1a, c, e**). Moreover, the biostimulant significantly increase the number of grains produced per spike under control conditions (NoSe) as shown in **Fig. V-1e**. At the Tiller, the biostimulant counteracted the negative effects caused by Se(VI) on all the

biomass parameters studied (**Fig. V-1b, d, f**) with values similar to the control ones (NoSe, NB) and improved as well the weight of both spikes and grain under the other Se treatments (**Fig. V-1b, d**). These results were expected since biostimulants are used to improve nutrient efficiency, abiotic stress tolerance and crop quality [20].

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 thus 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 (**Fig. V-1b, d, f**) up to values similar to those obtained in control plants.

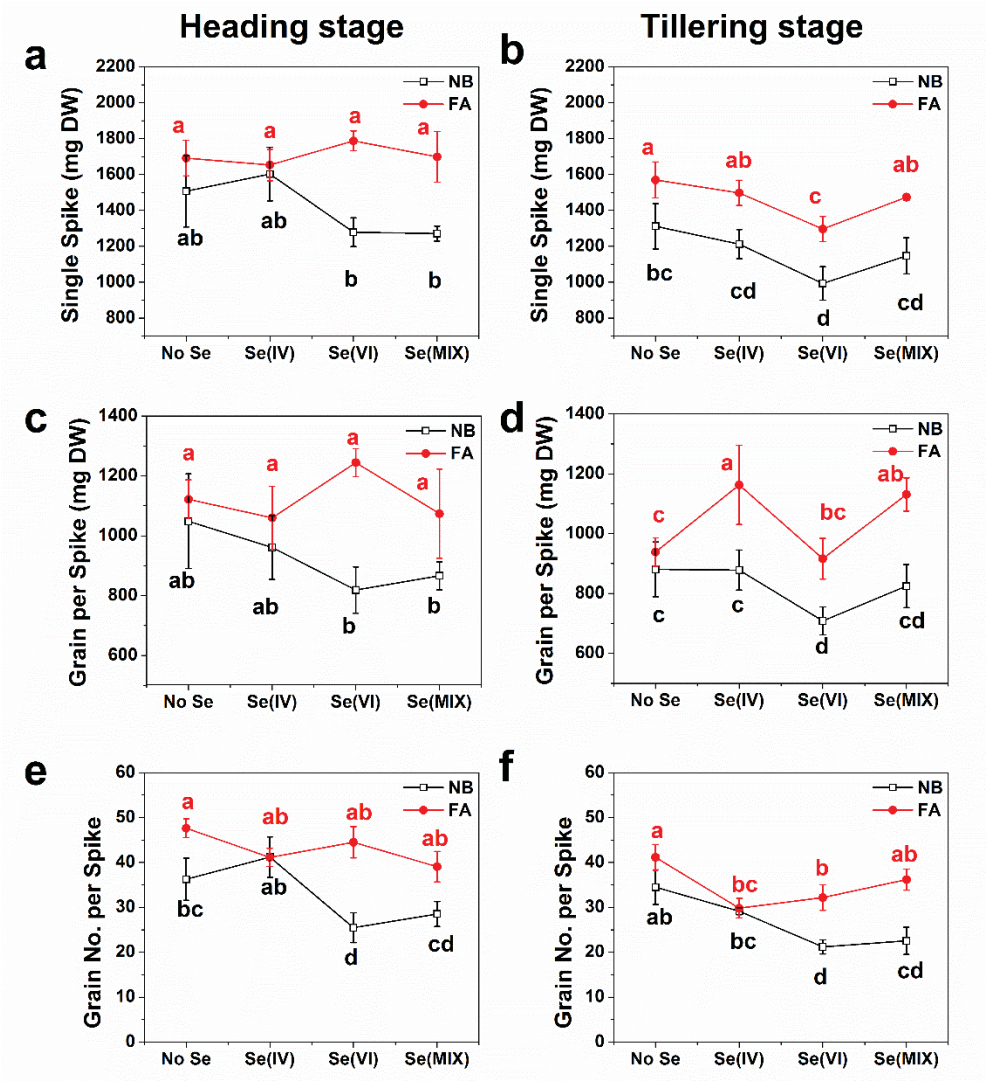

Figure V-1. Grain biomass parameters of *T. aestivum* plants grown under different Se treatments (selenite, selenate and mixture of both selenium species (10µM)) and biostimulant application (No biostimulant, Foliar Application) at different growth stages: Heading (a, c, e), Tillering (b, d, f). Results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

Total Selenium concentration in grain

Panels (a) and (b) of **Fig. V-2** show the total Se levels in grains for the different treatments applied. The results indicate that Se-biofortification of grains was achieved with values

within the range of 37-100 μg·g^{-1} DW and of 75-138 μg·g^{-1} DW for heading and tillering stages, respectively.

The Se concentration in grains obtained from plants exposed to Se(IV) achieved similar levels (90-100 μg.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 with values of 125-138 μg Se.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 [21,22] and not only due to a longer exposure time determined by the stage of application.

Although the application of biostimulants it is said to promote Se accumulation in wheat grain [15], the enhancement observed in our study was only statistically significant for Se(VI) treatment at the heading stage of application. Thus, the biostimulant does not increase Se accumulation in grains but influences other physiological parameters in the plants 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).

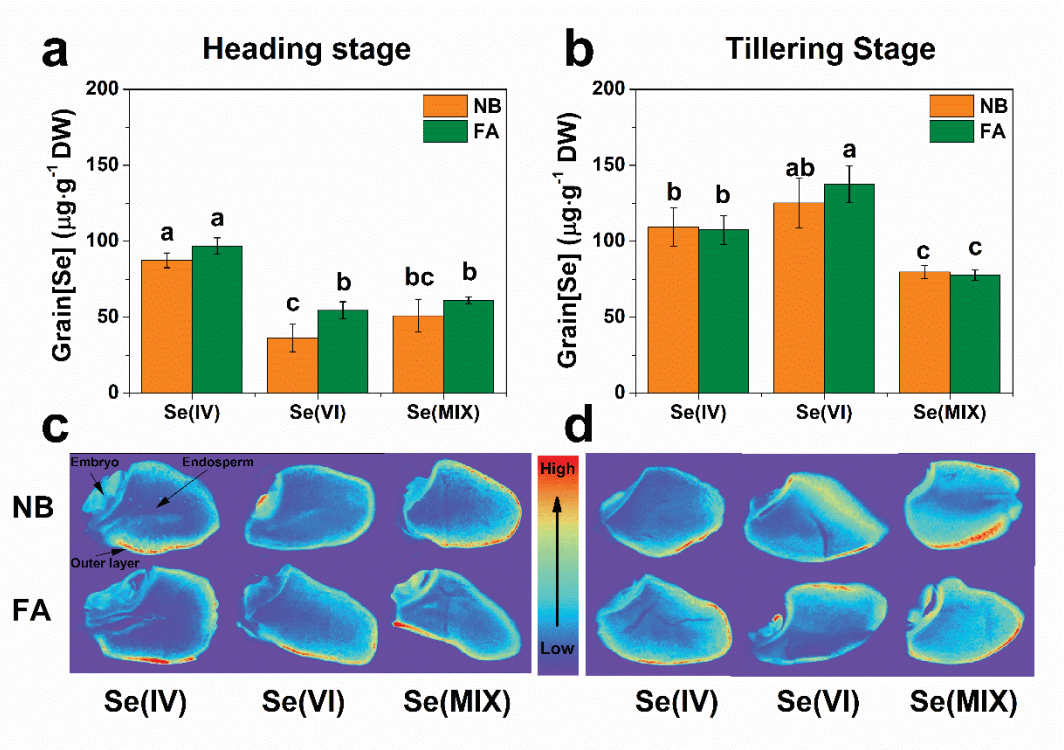

Figure V-2. Total Se concentration (top panels) and X-ray fluorescence mapping of Se (bottom panels) in wheat grain grown under different Se species (selenite, selenate and 1:1 mixture of selenium, and biostimulant application (No biostimulant, Foliar Application) at different growth stages: Heading (a, c), Tillering (b, d) . Total concentration displayed is mean ± SE (n=3). Different letters represent significantly differences among groups (LSD). Warmer colors in XRF maps indicate higher Se concentration.

Selenium and nutrient distribution in grain by using XRF mapping

Despite the valuable information extracted from the analysis of the total Se in 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 µ-XRF maps displayed in **Fig. V-2 c, 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 thus Se-proteins assembled from seleno-aminoacids are located there [23,24]. On the other hand, the images show much lower levels of Se accumulation in the endosperm which is mostly constituted by starch and contain with small fraction of fibers and proteins.

In addition, μ-XRF provides simultaneous information of the spatial distribution of several elements accumulated in the grain. This allows to get a direct insight of the placement of Se along with other nutrients**.** In our study, the XRF images for all the treatments show similar elemental distribution as the one displayed in **Fig. V-3** for the selenate applied at heading stage treatment (see **Fig. V-S2** for the rest of the treatments). The analysis of the XRF 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 [25]. This distribution is quite consistent, and it does not get affected by neither Se species supplied in the treatment or the application of plant biostimulants at different growth stage. Although outer layer is a reservoir of minerals in wheat grain [26], most of them are lost during the mechanical processing of wheat flour [27], which is not often consumed by people.

Tricolor RGB map helps to visualize the distribution patterns and co-localization of the nutrients and Se. As shown in **Fig. V-3**, K, Ca, Fe, Zn, Cu and Mn are mostly located in the embryo and outer layer covering the aleurone, seed coat and pericarp [25], hence, Se overlaps with them in some areas of outer layer. However, 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. This knowledge of grain tissue-specific element storage pattern can be useful in cereal processing and efficiency consuming [28]. Indeed, despite that the

outer layer is a reservoir of minerals in wheat grain [26], most of them are lost during the mechanical processing of wheat flour [27], which is not often consumed by people.

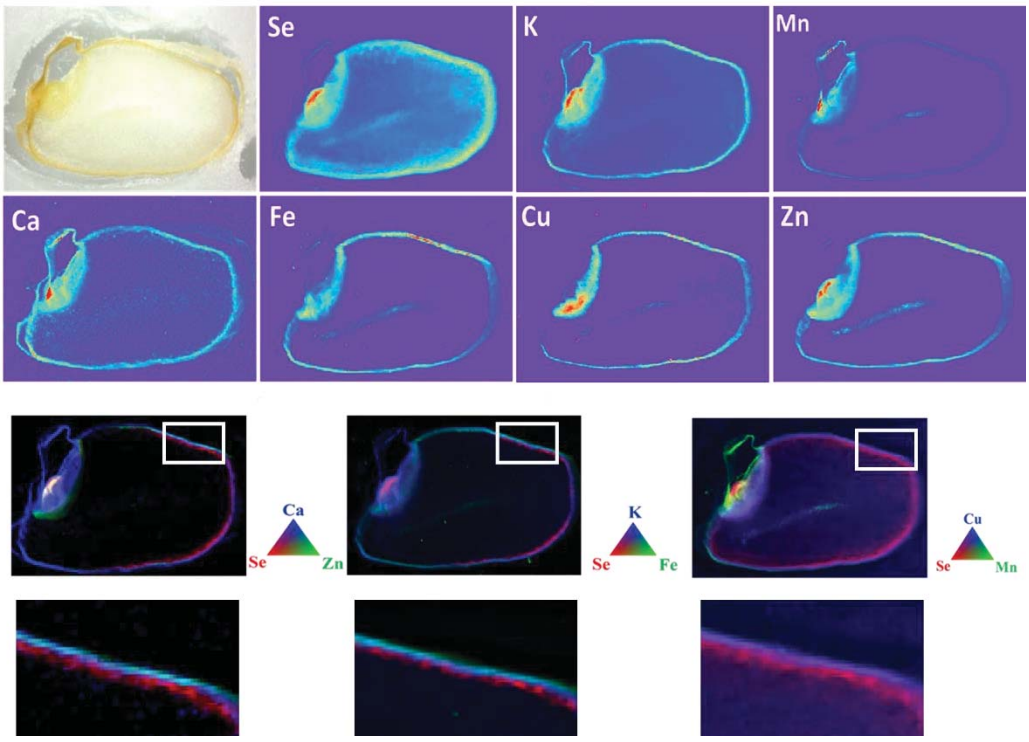

Figure V-3 μ-XRF elemental maps of wheat grains for selenate applied at the heading stage. Warmer colors indicate higher element concentration. The top two rows are the individual element distribution maps and optical microscope image (top left), the images at third row are the tricolor merged images and the images at bottom row are the zoomed in areas corresponding to the middle row. The colored triangle scales indicate the relative locations of elements color merged.

Selenium speciation in grain determined by μXANES

The level of Se accumulation, its localization in tissues within the grain together with other nutrients, and their occurrence in different chemical forms determine its dietary availability in cereals [25]. Hence, it is important to understand how the Se speciation might be affected when collocated with other elements present in the grain. 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 V-4b, c** shows the comparative for Se(MIX) treatment as a representative case of study. The spectra collected on the grains were compared with Se references samples (**Fig. V-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 spectral differences among the treatments suggest that the ratio among the Se species present varies.

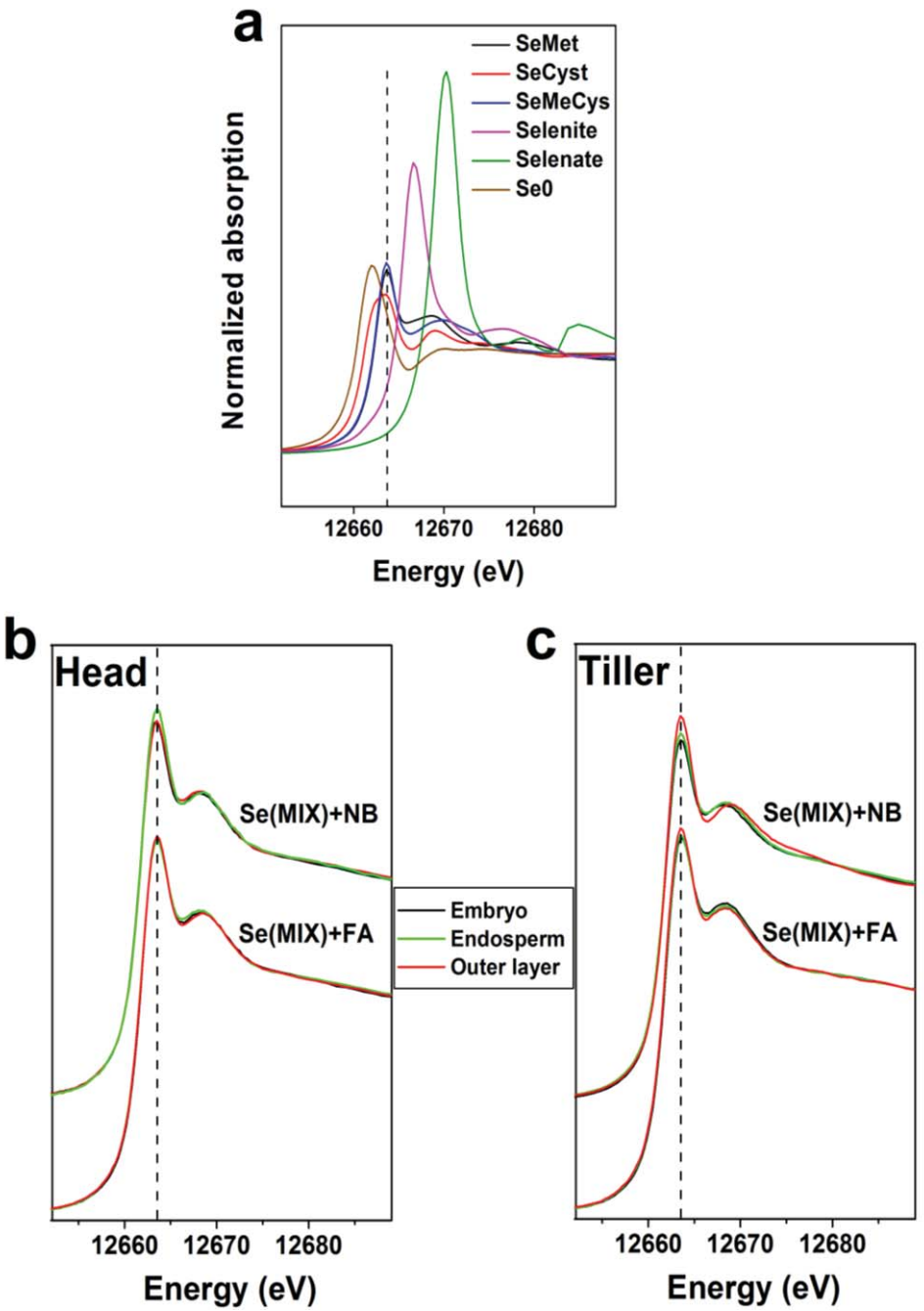

Figure V-4 Normalized Se K-edge XANES spectra of Se references (a) and wheat grain grown under Se(MIX) and biostimulant treatments applied at different growth stages: heading (Head) (b), and tillering (Tiller) (c). NB: No biostimulant, FA: Foliar application. The spectra have been shifted vertically for shake of comparison. Vertical line refers to the white-line contribution of species containing a C-Se-C bond (e.g. SeMet or SeMeCys).

To get a more quantitative information of the Se species present in the grain, a linear combination fitting (LCF) analysis has been performed using Se references as standards (**Fig. V-5**).

Characterizing the ratio of the Se species contained in the wheat grain to get an insight about the ratio of the seleno-amino acids formed is not only important to understand Se mechanism in plant, but also vital to human health since they have different both assimilation by the human body and their functions for specific health benefits. It has been reported that 25 selenoproteins have SeCys at their active center taking part in human biochemistry activities, such as anti-oxidation [29]. Equally, Se supplementation of SeMet is effective against Hashinoto's thyroid disease [30].

The values obtained from the LCF analysis have been included in Tables S1 and S2 of the supporting information. **Figure V-5a** reports the ratio between inorganic and organic species for the NB treatment applied at the heading and tillering stages. These results confirm that organic Se species are the main component in Se-biofortified wheat grains. FA treatment did not significantly influence this ratio (see **Fig. V-S3**). They are in agreement with previous studies reporting that the organic Se species are the main Se species present in wheat grain [31,32]. This comparative also shows that the application of Se at different stages of the plant growth affects the proportion of organic Se in wheat grains. The amount of organic Se species are always larger than 90% when the treatment is applied at the tillering stage, whereas for the heading stage they are lower than 80% in most of the cases. This indicates that Se exposure period are important parameters in the conversion of inorganic Se to organic Se, even in those cases for which the Se enrichment achieved are in the same level (e.g. Se(IV) treatment).

A better insight in the composition is achieved when considering each independent Se species included in the LCF analysis. As shown in (**Fig. V-5b, c**), Se species containing a C-Se-C bond (SeMet and SeMeCys) are the main compounds distributed in the different parts of the grain when the Se treatment is applied at the heading stage. In addition, grains from plants under biostimulant treatment (FA) seems to accumulate more C-Se-C amino acid and elemental Se in comparison with the control group (NB) when Se is applied at heading stage (**Fig. V-5b**) even though the total amount of organic species remains very similar for both treatments. Hence, the amount of C-Se-Se-C (SeCyst) amino acid in NB is larger than in FA ones. However, when Se is applied at the tillering stage (**Fig. V-5c**) the amount of C-Se-C species is lower than for the heading stage.

Although both C-Se-C species and SeCys both can be incorporated into proteins in place of Met and SeCys leading to toxicity, C-Se-C species have less harmful effects [33], since the SeCys into protein could interfere with the formation of disulfide bridges affecting tertiary structure of S-proteins 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 grain is that total Se concentration decreases together with the total organic Se, and there is an increase of C-Se-C respect to the total organic Se at the heading stage group. Although FA group contains more C-Se-C and elemental Se than NB treatment in the heading stage group, the contribution of FA in the Se tolerance is too narrow to be concluded.

By comparing **Fig. V-5b** and **Fig. V-5c**, it can be noticed that Se(0) is only detected in the heading stage of application and it is negligible in the tillering one. Se(0) is one of the product derived from SeCys, via the action of a selenocysteine lyase (SL). Elemental Se is comparatively innocuous since the potential Se detoxification mechanism [34,35]. This indicates that when Se is applied at the heading stage it could minimize the Se toxicity in

wheat. Plant has a complex defense mechanism to maintain the yield under stress. In the heading group, Se as abiotic stress applied at grain spike just appearing, it probably stimulates the expression of SL in order to enhance Se tolerance and maintain the growth cycle. Thus, Se(0) was only found in the heading stage application.

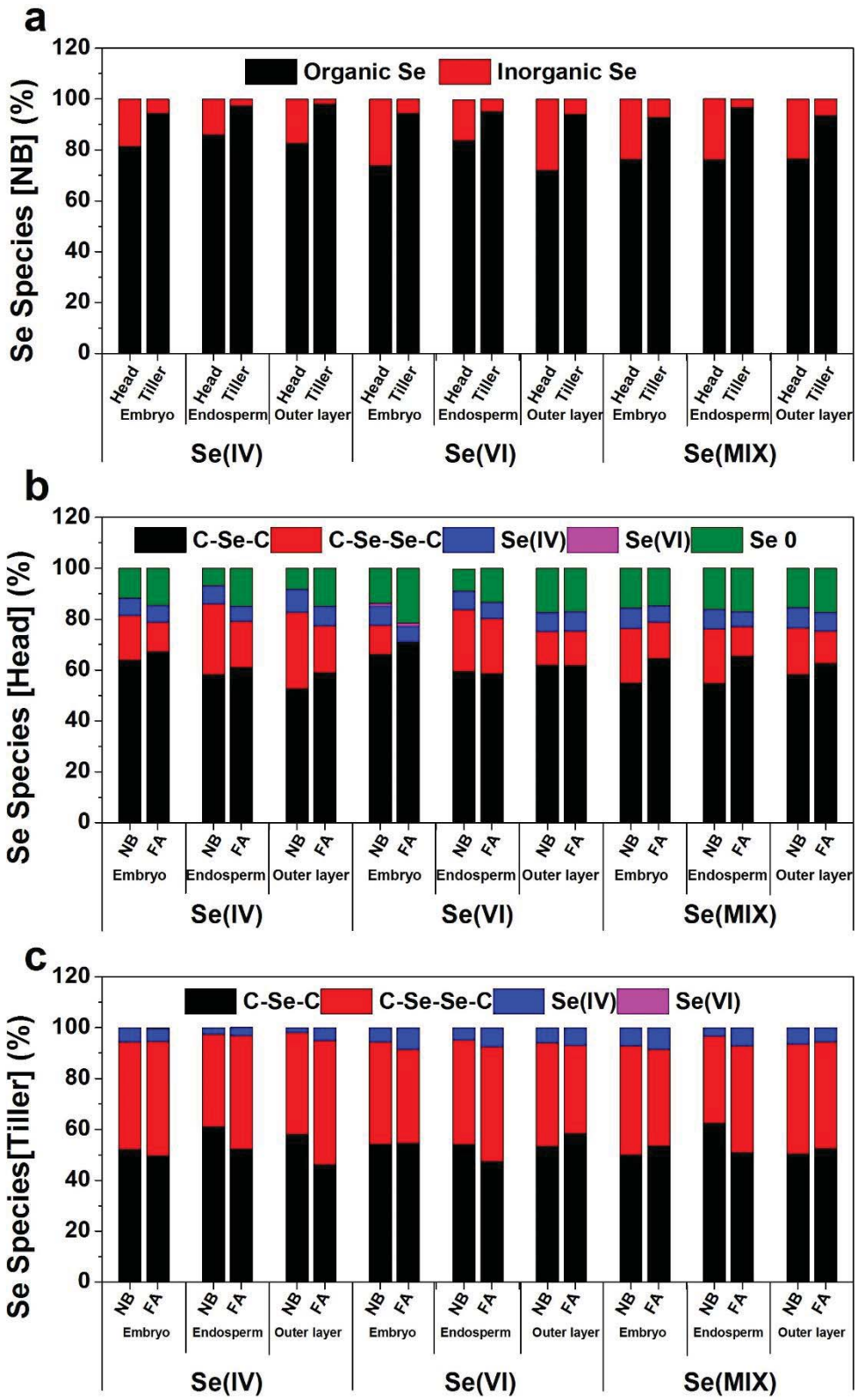

Figure V-5 Results from the linear combination fitting analysis of the μXANES spectra collected at different parts of the wheat grain: (a) Ratio between organic and inorganic Se species at different Se application stages (heading (Head) or tillering (Tiller)) without

biostimulant (NB), (b and c) Se speciation when Se was applied at the heading stage (Head) or at the tillering stage (Tiller) without plant biostimulant application (NB) and with plant biostimulant foliar application (FA). C-Se-C refer to Selenomehtionine and Se-Methylselenocysteine. C-Se-Se-C refer to SelenoCystine. See text for details.

Conclusions

Our results show that the application of the plant biostimulant did not promote Se accumulation in grains but had an influence on other physiological parameters of the plants that enhanced grain performance counteracting the Se-induce toxicity promoted by an early Se exposure probably due to the enhanced catalytic influence of the Mo species from biostimulant on the physiology of vegetal cells through their mitochondria activity. In this context the biostimulant had a key role in enhancement 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. Only Se in the form of Se(VI) supplied at the tillering stage caused negative effects on wheat grain performance due to the achievement of the highest Se levels. Se-biofortification of wheat grain was achieved in both Se stages of application whereas any Se treatment applied at the heading stage seems to minimize Se induced toxicity.

Our study shows that organic Se species are the main species found in wheat grain and that they are collocated 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 or the application of plant biostimulants at different growth stage. 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 plants treated at the tillering stage contains higher ratio of C-Se-C and lower C-Se-Se-C than grain treated at heading stage. The last having a ratio of C-Se-C and C-Se-Se-C almost equal.

These results have allowed us to get valuable information about Se effective utilization, providing a fundamental reference on soil cultivation. Because several factors in soil including pH, moisture and minerals levels would affect Se absorption by plant root, a further study on Se effective utilization in soil is required. The mechanism for the application of plant biostimulant to crops needs further study in order to reduce the stress suffered by wheat plants and increase the viability and development of those cultivations, thus increasing the crop yield. Although wheat grain is a reservoir of minerals mostly accumulated in outer layer, most of them are lost during the mechanical processing of wheat flour, is not often consumed by people. Thus, the crop consume way also needs to be reconsidered.

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Supplementary information

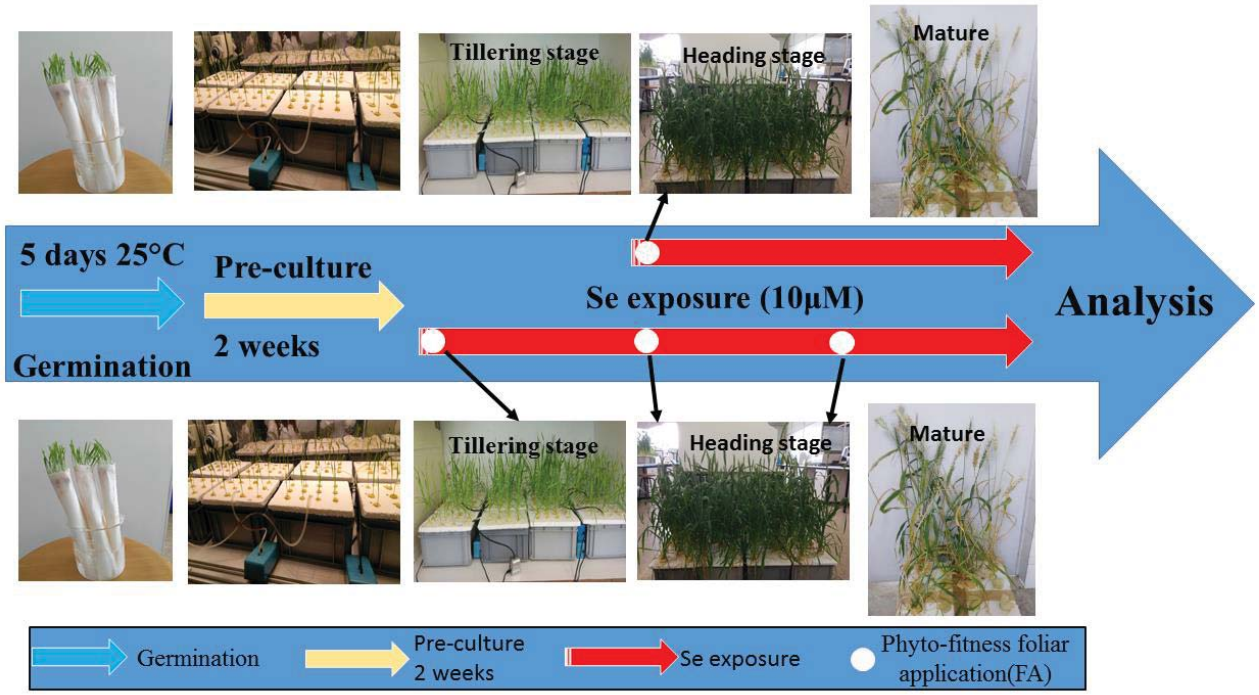
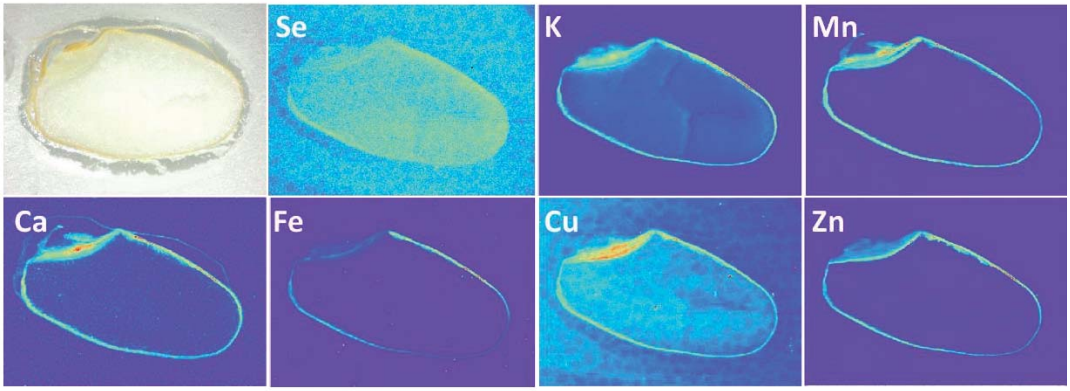


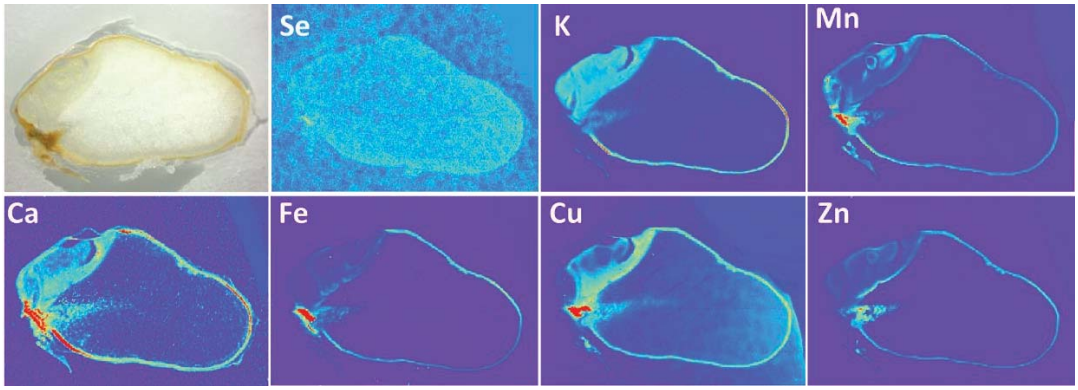
Figure V-S1 The schematic diagram showing the culture of experimental wheat samples

Heading stage application

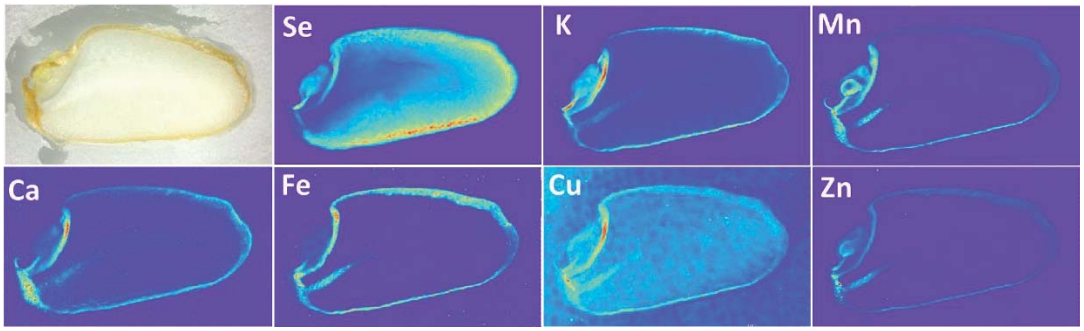
No Se_NB



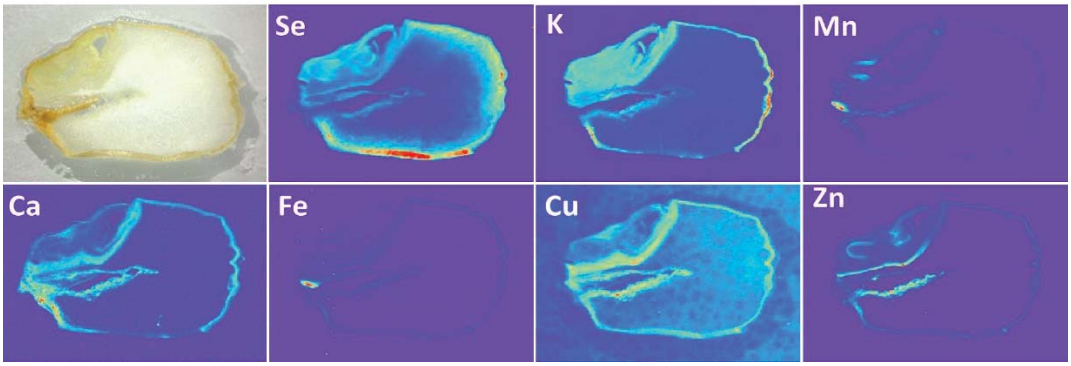
No Se_FA



Se(IV)_NB



Se(IV)_FA



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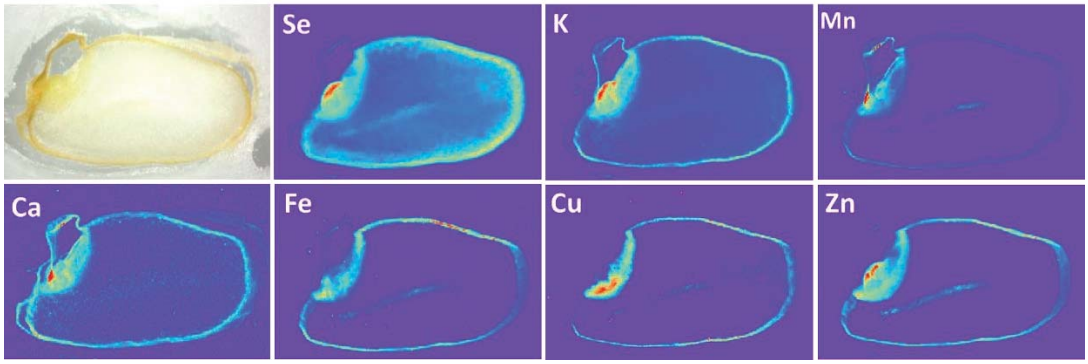

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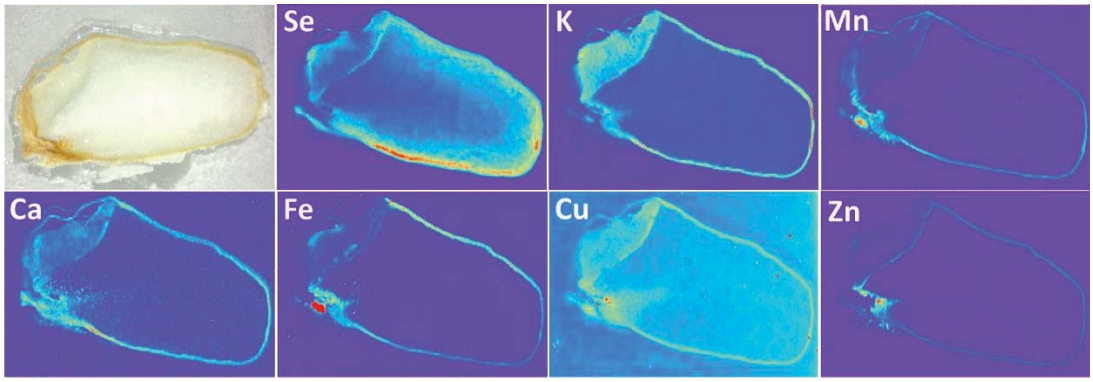
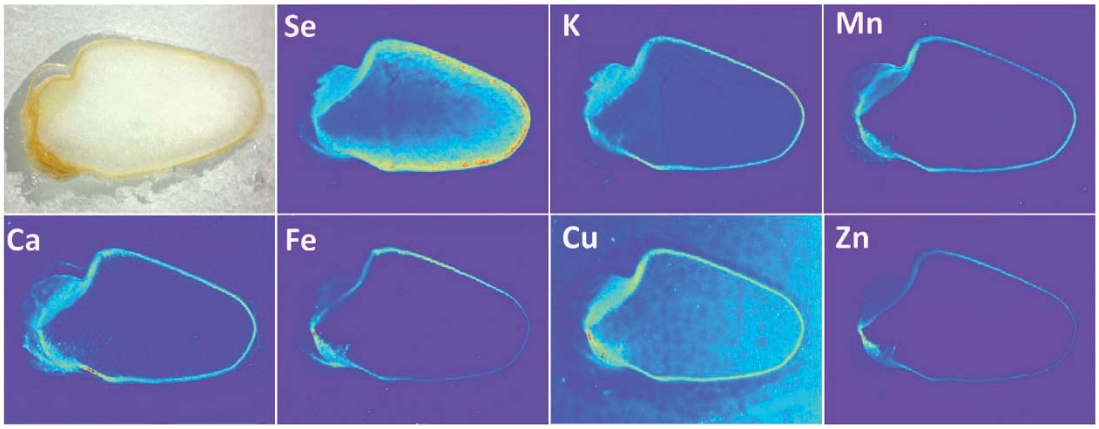
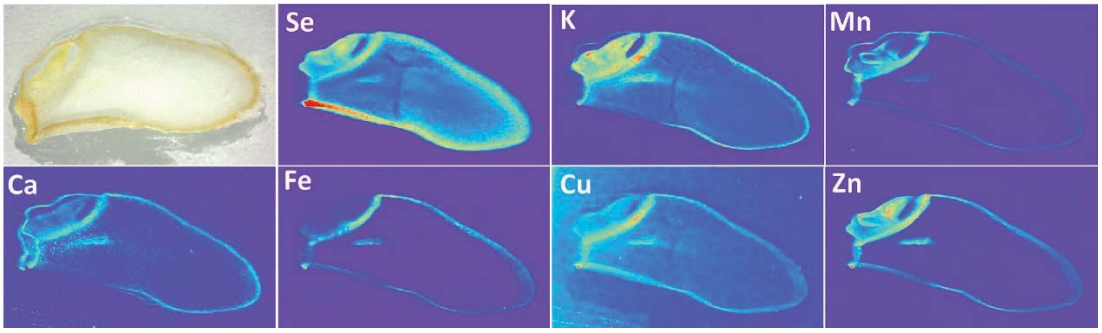


Se(MIX)_NB



Se(MIX)_FA



Tillering stage application

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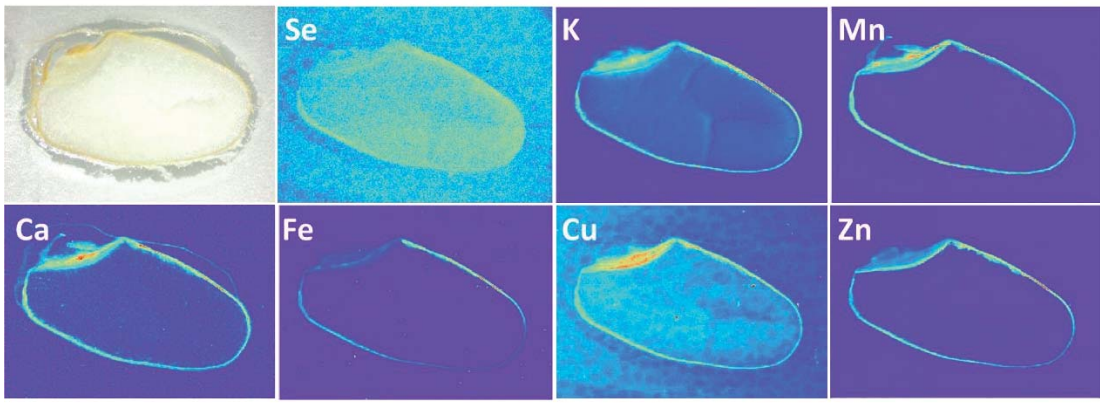
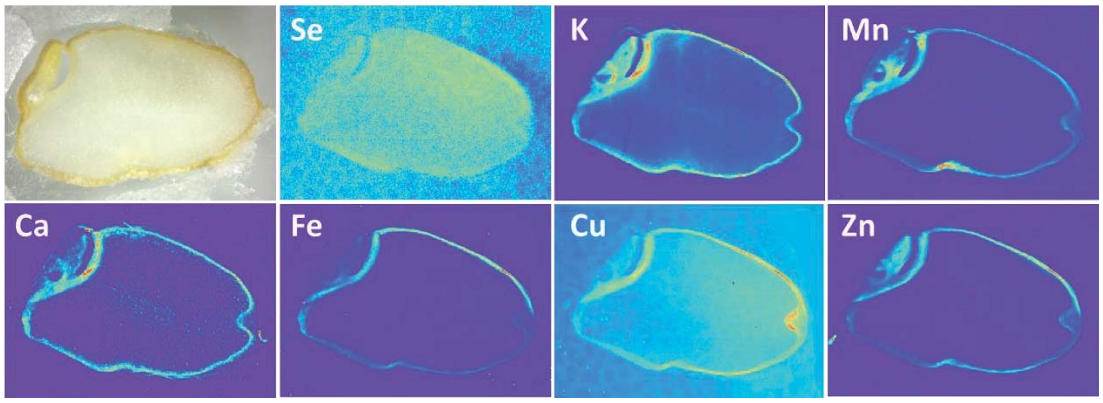
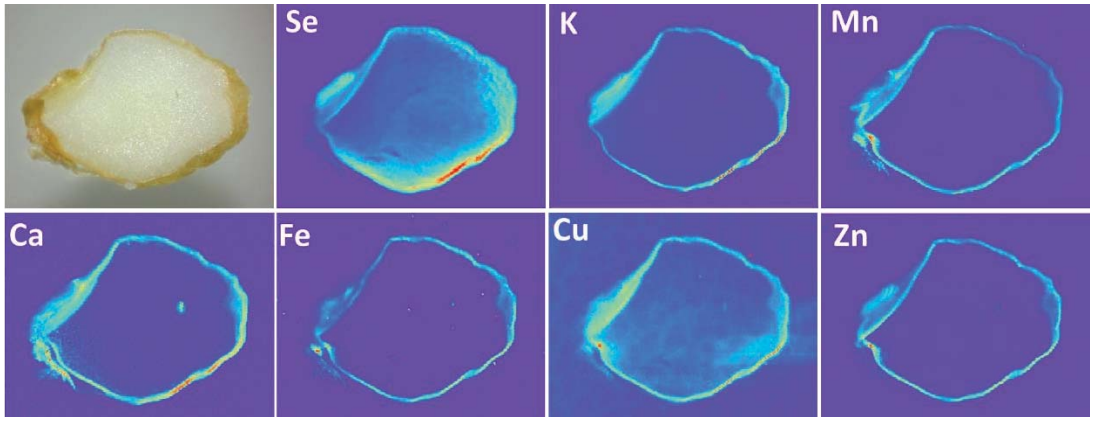


No Se_FA



Se(IV)_NB



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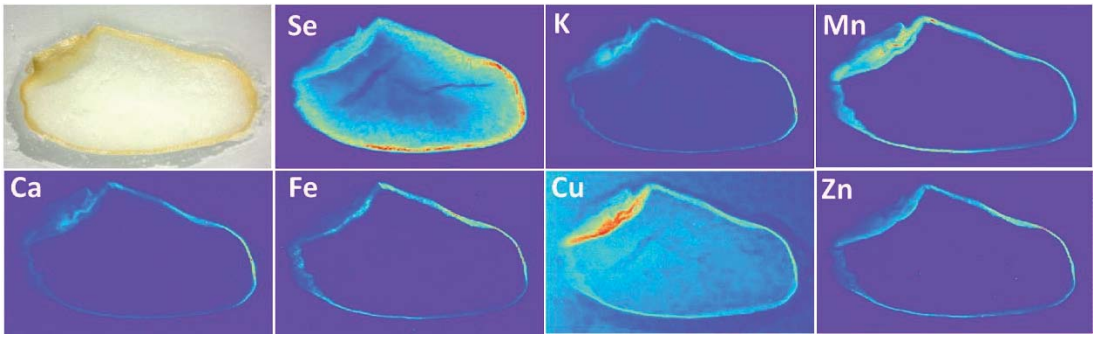

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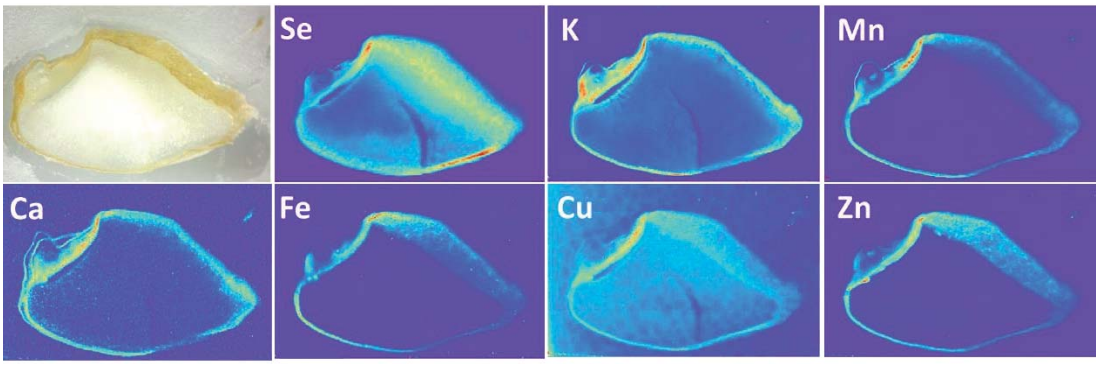
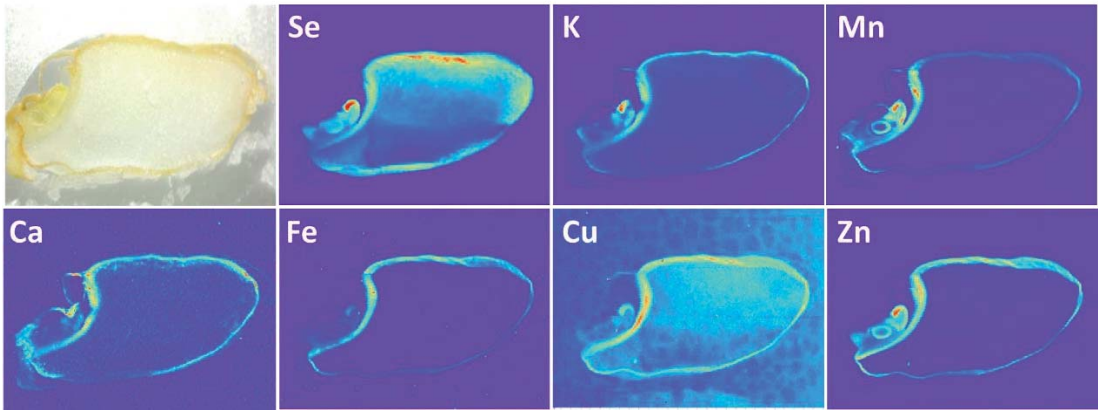
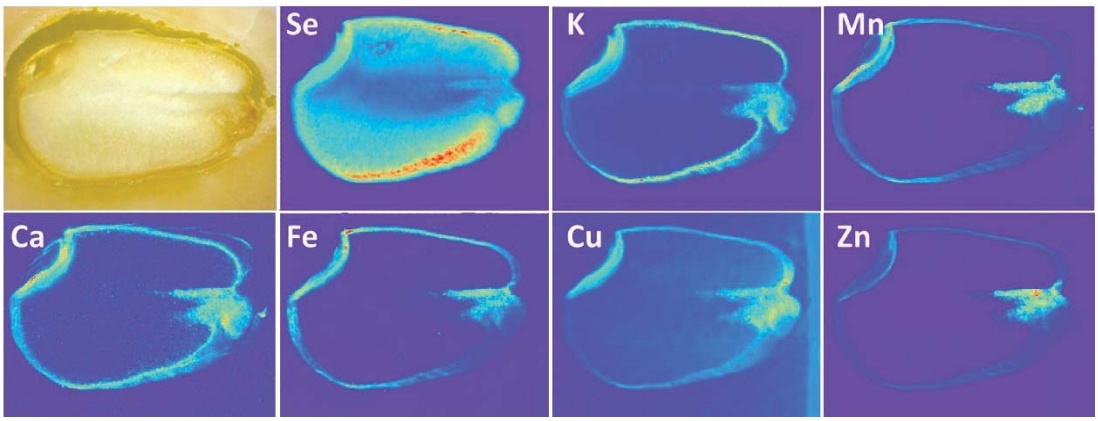


Se(VI)_FA



Se(MIX)_NB



Se(MIX)_FA

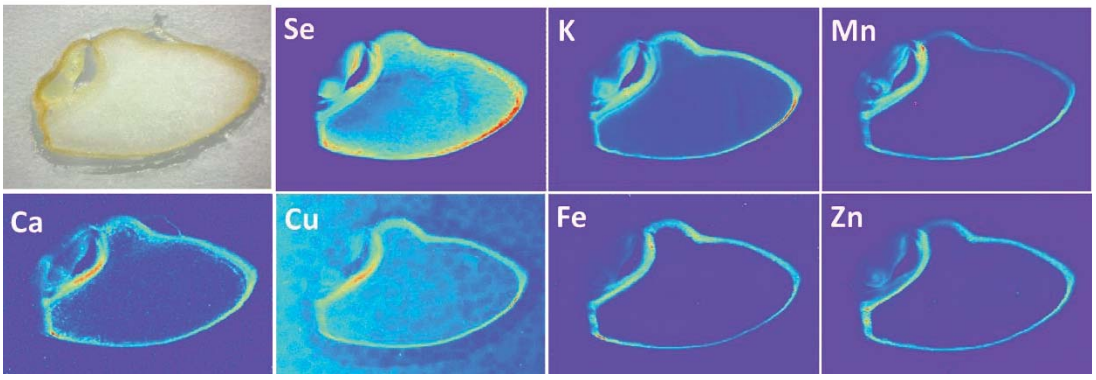


Figure V-S2 μ-XRF elemental maps of wheat grains.

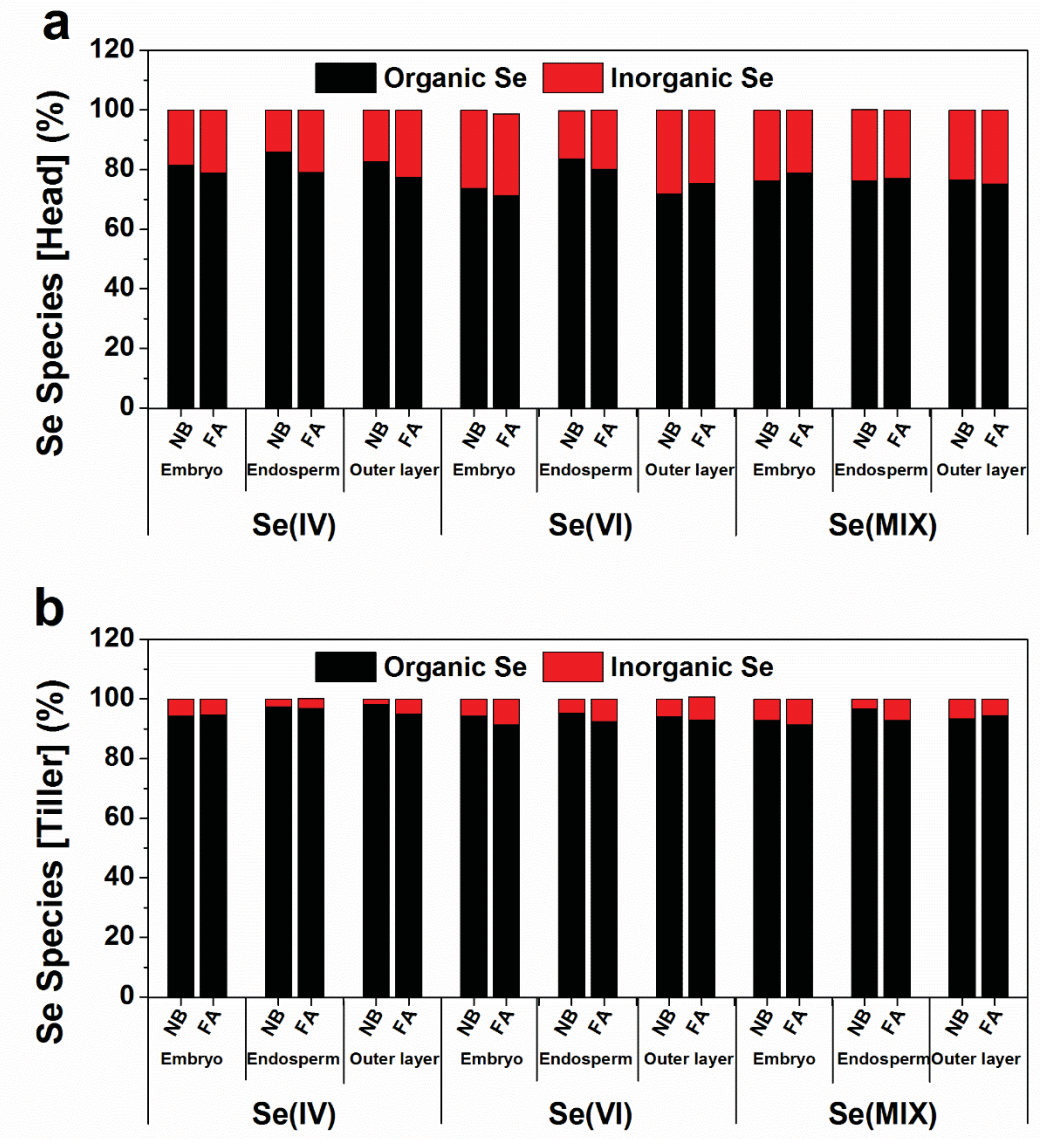


Figure V-S3 Results from the linear combination fitting of the μXANES spectra collected at different parts of the wheat grain: (a), (b) Ratio between organic and inorganic Se species for different selenium application and biostimulants methodology (Head or Tiller), respectively. Without plant biostimulant application (NB) comparable with plant biostimulant foliar application (FA)NB refer to no biostimulant, Tiller refer to Tillering stage, Head refer to Heading stage.

Table V-S1 Results from linear combination fitting Se K-edge X-ray absorption spectra obtained from wheat grain (Head application). C-Se-C refers to SeMet, MeSeCys; C-Se-Se-C refers to SeCyst. Se(IV) and Se(VI) refer to selenite and selenate contributions. The weight of each component is expressed as % of the total. The value in parenthesis is the uncertainty calculated as the standard deviation. See text for details.

Selenium Treatments	Grain tissue	Biostimulant treatment	R-factor [a]	C-Se-C	C-Se-Se-C	Se(IV)	Se(VI)	Se(0)
Se(IV)	Embryo	NB	0.0015	63.9 (0.026)	17.5 (0.036)	6.8 (0.047)	-	11.8 (0.016)
		FA	0.0017	67.1 (0.167)	11.7 (0.037)	6.6 (0.167)	-	14.6 (0.017)
	Endosperm	NB	0.0028	59.6 (0.037)	26.4 (0.045)	7.0 (0.007)	-	7.0 (0.007)
		FA	0.0017	61.0 (0.048)	18.1 (0.037)	5.9 (0.060)	-	15.0 (0.017)
	Outer layer	NB	0.0048	52.6 (0.048)	30.2 (0.063)	9.0 (0.010)	-	8.2 (0.029)
		FA	0.0019	58.9 (0.029)	18.4 (0.040)	7.7 (0.052)	-	15.0 (0.018)
Se(VI)	Embryo	NB	0.0020	73.8 (0.011)	-	6.2 (0.005)	1.5 (0.016)	18.5 (0.008)
		FA	0.0037	72.6 (0.036)	-	6.0 (0.007)	-	21.4 (0.010)
	Endosperm	NB	0.0029	59.4 (0.037)	24.6 (0.072)	7.3 (0.007)	-	8.7 (0.022)
		FA	0.0016	58.7 (0.027)	21.4 (0.040)	6.5 (0.005)	-	13.4 (0.016)
	Outer layer	NB	0.0020	69.0 (0.011)	3.5 (0.019)	7.4 (0.008)	1.1 (0.003)	19.0 (0.015)
		FA	0.0016	61.8 (0.027)	13.5 (0.036)	7.6 (0.050)		17.1 (0.016)
Se(MIX)	Embryo	NB	0.0013	54.9 (0.036)	21.5 (0.033)	8.1 (0.005)	-	15.5 (0.015)
		FA	0.0017	64.5 (0.027)	14.3 (0.037)	6.5 (0.027)	-	14.7 (0.037)
	Endosperm	NB	0.0018	54.7 (0.028)	21.5 (0.045)	7.6 (0.006)	-	16.2 (0.017)
		FA	0.0016	65.4 (0.155)	11.6 (0.036)	5.9 (0.005)	-	17.1 (0.016)
	Outer layer	NB	0.0017	58.3 (0.027)	18.1 (0.037)	8.2 (0.006)	-	15.4 (0.047)
		FA	0.0017	62.7 (0.043)	12.5 (0.037)	7.5 (0.005)	-	17.3 (0.017)

[a] R-factor is a measure of the mean square sum of the misfit at each data point which gives an indication of the goodness of fit. It is defined as: R-factor $= \frac{\sum(data-fit)^2}{\sum data^2}$, where the sums are over the data points in the fitting region.

Table V-S2 Results from linear combination fitting Se K-edge X-ray absorption spectra obtained from wheat grain (Tiller application). C-Se-C refers to SeMet, MeSeCys; C-Se-Se-C refers to SeCyst. Se(IV) and Se(VI) refer to selenite and selenate contributions. The weight of each component is expressed as % of the total. The value in parenthesis is the uncertainty calculated as the standard deviation. See text for details.

Selenium Treatment	Grain tissue	Biostimulants Treament	R-factors [a]	C-Se-C	C-Se-Se-C	Se(IV)	Se(VI)
Se(IV)	Embryo	NB	0.0037	52.2 (0.084)	42.1 (0.022)	5.7 (0.122)	-
		FA	0.0048	49.7 (0.029)	44.9 (0.033)	5.0 (0.09)	0.4 (0.005)
	Endosperm	NB	0.0013	61.1 (0.052)	36.3 (0.075)	2.6 (0.006)	-
		FA	0.0026	52.2 (0.069)	44.5 (0.018)	3.3 (0.008)	-
	Outer layer	NB	0.0018	58.1 (0.075)	40.0 (0.016)	1.9 (0.007)	-
		FA	0.0020	46.2 (0.062)	48.7 (0.090)	5.1 (0.007)	-
Se(VI)	Embryo	NB	0.0037	54.3 (0.088)	40.0 (0.022)	5.7 (0.122)	-
		FA	0.0047	54.6 (0.10)	36.7 (0.145)	8.7 (0.011)	-
	Endosperm	NB	0.0012	54.0 (0.049)	41.1 (0.013)	4.9 (0.006)	-
		FA	0.0032	47.4 (0.09)	45.0 (0.021)	7.6 (0.009)	-
	Outer layer	NB	0.0010	53.3 (0.043)	40.7 (0.011)	6.0 (0.005)	-
		FA	0.0114	58.4 (0.18)	34.6 (0.041)	7.0 (0.018)	-
Se(MIX)	Embryo	NB	0.0021	50.1 (0.038)	42.7 (0.016)	7.2 (0.007)	-
		FA	0.0032	53.5 (0.104)	37.9 (0.021)	8.6 (0.009)	-
	Endosperm	NB	0.0012	62.4 (0.063)	34.3 (0.013)	3.3 (0.006)	-
		FA	0.0040	50.8 (0.103)	41.9 (0.023)	7.3 (0.01)	-
	Outer layer	NB	0.0011	50.4 (0.115)	43.0 (0.012)	6.6 (0.005)	-
		FA	0.0034	52.4 (0.221)	42.0 (0.022)	5.6 (0.01)	-

[a] R-factor is a measure of the mean square sum of the misfit at each data point which gives an indication of the goodness of fit. It is defined as: R-factor $= \frac{\sum(data-fit)^2}{\sum data^2}$, where the sums are over the data points in the fitting regi

Conclusions

From results previously reported in Chapter Ⅲ, Ⅳ and Ⅴ, the following conclusions can be summarized as below:

Se species effect on short-term plants

1. Inorganic Se speciation, Se(IV) and Se(VI) has different metabolic pathways in plant tissues. Se(VI) is taken up actively via sulphate transporter, Se(IV) is taken up by phosphate transporter. Se(IV) is rapidly reduced to organic Se in roots and has limited translocation to shoots. In contrast, Se(VI) reduction is slower, Se(VI) reduction to Se(IV) being the limiting step in Se metabolism. Se(VI) is readily absorbed in roots and transferred to shoots.
2. Organic Se is the main Se species found in Se(IV) treatment, while the lower amount of organic Se and higher amount of inorganic Se was found in Se(VI)-treated wheat plants. The levels in Se(MIX)-treated plants are the average between Se(IV) and Se(VI) as expected.
3. The shoot levels of the micronutrients Cu, Zn, Mo are decreased by Se(IV). This could be due to a reduction in water content and thus in mineral compounds uptake.
4. Wheat plants alkalify the nutrient solution in control and all Se treatments. Se species do not affect the pH of the xylem sap. Translocation of mineral elements is a key process to control Se and nutrient accumulation in wheat aerial part.
5. The pattern of S accumulation was similar to that found for Se under the same experimental conditions, especially in shoot. Se species supplement affected S levels in plants. Indeed, Se(VI) caused a significant increase of S in shoots by stimulating sulfate transporter expression

6. Titration technique could act as a supplementary and effective tool for plant signaling studies with the advantages of requiring simple sample pretreatment and being low-cost.

Se species effect on long-term plants

7. 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. Only Se in the form of Se(VI) supplied at the tillering stage caused negative effects on wheat grain performance due to the achievement of the highest Se levels in them. Se-biofortification of grain was achieved both at heading and tillering stages of Se application, whereas any Se treatment applied at the heading stage seems to minimize Se-induced toxicity.

8. Organic Se are the main species found in wheat grain and that they are collocated 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 or the application of plant biostimulants at different growth stage. The amount of organic Se species are always larger than 90% when the treatment is applied at tillering stage, whereas for heading stage they are lower than 80% in most of the cases. Plants at tillering stage of Se application contains higher ratio of C-Se-C and lower C-Se-Se-C than plants at heading stage of Se application that the ratio of C-Se-C and C-Se-Se-C are almost equal.

Plant biostimulants effect on short-term plant

9. The foliar application of the biostimulant (FA) did not modify the plant biomass. On the other hand, when the biostimulant was root applied (RA) a significantly increased of the root biomass in all treatments as well as the shoot biomass of plants exposed to

Se(VI) and Se(MIX) were observed.

10. In roots, the FA treatment had no influence on IAA levels while RA treatment increased IAA concentration of plants exposed to Se(IV) and Se(MIX) above control ones (NB, No Se). Both biostimulant modes of application caused a severe reduction of IAA levels in shoots. It seems that the biostimulant may inhibit the basipetal transport of auxin from stem tip to root.

11. The biostimulant accelerated the translocation of Se from roots to shoots in the presence of Se(VI) and Se(MIX), and only had an influence modified on the Se speciation species only in the roots, not in shoots. To some extent, the biostimulant can help plants to grow under Se exposure.

Plant biostimulants effect on long-term plant

12. The application of the plant biostimulant did not promote Se accumulation in grains but had an influence on other physiological parameters of the plants that enhanced grain performance counteracting the Se-induce toxicity promoted by an early Se exposure.

13. In our study, the biostimulant had a key role in enhancement 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.