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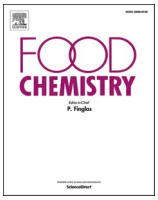

Liposomes as selenium nanocarriers for foliar application to wheat plants: A biofortification strategy

Marcia Viltres-Portales [a,b,1], María-Jesús Sánchez-Martín [a,*,1], Roberto Boada [a], Mercè Llugany [c], Manuel Valiente [a]

[a] GTS Research Group, Department of Chemistry, Faculty of Science, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[b] Institute of Materials Science and Technology, Universidad de La Habana, Zapata y G, Vedado, Plaza, 10400 La Habana, Cuba
[c] Plant Physiology Group (BABVE), Faculty of Biosciences, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

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ABSTRACT

In the present work, liposomes have been used as nanocarriers in the biofortification of wheat plants with selenium (Se) through foliar application. Liposomal formulations were prepared using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and Phospholipon®90H (P90H) (average size <100 nm), loaded with different concentrations of inorganic Se (selenite and selenate) and applied twice to the plants in the stage of vegetative growth. Liposomes enhanced Se uptake by wheat plants compared to direct application. The highest Se enrichment was achieved using the phospholipid DPPC and a concentration of 1000 μmol·L^{-1} of Se without affecting the biomass, chlorophylls, carotenoids, and the concentration of mineral nutrients of the plants. The chemical speciation of Se in the plants was further investigated by X-ray absorption spectroscopy (XAS). The results from XAS spectra revealed that most of the inorganic Se was transformed to organic Se and that the use of liposomes influenced the proportion of C-Se-C over C-Se-Se-C species.

1. Introduction

Selenium (Se) is considered an essential micronutrient for human health. At least 25 selenoproteins have been identified with key roles in antioxidant systems, hormone regulation, immunology, male fertility, resistance to viral infections, and cancer prevention (Jitaru et al., 2009; Pyrzyńska, 2002; Vinceti, Filippini, & Wise, 2018). However, this element has a double-edged effect in terms of essentiality and toxicity: selenium concentrations below the recommended daily intake of 55 μg Se/day for adults may produce non-proper selenoproteins' metabolism and function, and higher than 400 μg Se/day may cause serious diseases (Kieliszek, 2019; Kipp et al., 2015). So far, it has been estimated that 1 billion of the global population have a scarce intake of Se in their diets (Deng, Liao, Zhao, Qin, & Liu, 2022; Tan, Mo, Lau, & Xu, 2018). Therefore, the bioavailability and consumption of Se by the population is a current concern (Rayman, 2020; Shahid et al., 2018). This alarming situation is caused by the low concentration of Se in soils and its heterogeneous distribution with the exacerbation of climate change (Jones et al., 2017).

Biofortification of edible crops through agronomic practices has been addressed as an alternative to decrease the Se shortage in humans caused by Se-deficient soils (Hossain et al., 2021; Schiavon, Nardi, Dalla Vecchia, & Ertani, 2020). Current research focuses on developing the most effective Se application technologies for crop biofortification that reduce the long-term impact on agricultural soils and do not affect plant nutrient content. Among several strategies, foliar applications allow easy and rapid application of nutrients and fertilizers by spraying them directly onto the leaves and stems of plants, where they are absorbed (Delaqua, Carnier, Berton, Corbi, & Coscione, 2021; Di et al., 2023). This approach is ideal for the application of small amounts of nutrients as it has an immediate effect on plants, requires less use of the product and is not dependent on soil characteristics (Ferrari et al., 2021). However, some disadvantages of foliar treatments are lower uptake and mobility of nutrients and fertilizers compared to soil applications. In addition, the application of high concentrations can cause a burning effect and the cost of carrying out multiple applications can be expensive (Niu, Liu, Huang, Liu, & Yan, 2021).

Nanoparticle engineering has created nanocarriers as delivery

* Corresponding author.
E-mail address: mariajesus.sanchez@uab.cat (M.-J. Sánchez-Martín).
[1] Shared co-first authorship.

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systems for active substances in medical, veterinary, and agricultural applications over the last 30 years. Nanocarriers are used to deliver, among others, small molecules, peptides and proteins, nucleic acids, or combinations of these. Small molecules are low-molecular-weight organic compounds with beneficial biological activities, such as anticancer drugs, antibiotics, fertilizers, and pesticides (Chariou, Ortega-Rivera, & Steinmetz, 2020). Nanospheres, nanocapsules, liposomes, micelles, cell ghosts, lipoproteins, and other pharmaceutical nanocarriers are widely used for experimental (and clinical) delivery of therapeutic and diagnostic substances. For agricultural applications, many opportunities have been created by using nanotechnology for the safe application of conventional pesticides (Nuruzzaman, Rahman, Liu, & Naidu, 2016).

Liposomes are nanoparticles that offer many advantages over other drug delivery systems: their main characteristics is their biocompatibility and biodegradability, allowing them to be used in medicine for the delivery of a wide range of active products, including small molecules, proteins, and nucleic acids. They are spherical vesicles composed of one or more concentric bilayers of phospholipids with an aqueous core, with particles sizes ranging from 20 nm to several microns. Liposomes are very suitable for entrapping both lipophilic (in the lipidic membrane) and hydrophilic compounds (in the aqueous core). They have potential applications in agriculture, as their nano-sized particles can enhance the efficacy of agrochemicals (such as pesticides, fertilizers, and growth hormones), and deliver them in a controlled and targeted manner (Farshchi, Azizi, Teymouri, Nikpoor, & Jaafari, 2021; Karny, Zinger, Kajal, Shainsky-Roitman, & Schroeder, 2018) by fusioning with the plant cell membranes and releasing their content. However, for agrochemicals' delivery applications in agriculture, liposomal formulations require narrow size distributions (<200 nm) to easily penetrate the plant cell barriers and long-term stability (Mozafari, 2005). The charge of the liposomes also affects the penetration in plant leaves. Previous studies have shown that negatively charged particles are more likely to be transported through vascular tissues, while positively or neutrally charged particles can cross the cell membrane by endocytosis and are more favourable for accumulation on the plant vascular system and therefore not be transported (Hong et al., 2021). Another important aspect to contemplate is that liposomes are considered as environmentally friendly, non-toxic substances, biocompatible and biodegradable to soils or the environment in general, they have been used in the bioremediation of soil contaminants such as oil and petroleum (Barenholz et al., 2003; Gomez-Guzman et al., 2023). Also, the 47% of nanocarriers most frequently approved in clinical trials for medical and veterinary applications (regulated by the Food and Drug Administration in the United States and the European Medicines Agency in Europe) are based on liposomes, in part because of their safety (Chariou et al., 2020).

It is important to note that liposomal formulations can be expensive to produce due to the cost of the phospholipids and other raw materials, which may limit their use in agricultural applications, where high amounts are needed to cover crop fields. This may explain why there are only a few examples in this area, despite the potential for large number of phospholipids to be explored. In this sense, two phospholipids were used as nanocarriers in the obtention of liposomes' formulations to encapsulate selenium for the biofortification of wheat plants. DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) is a saturated phospholipid that have been quite studied for encapsulation and controlled release of drugs in clinical studies, and as a model system to mimic the cell membrane considering its biocompatibility and biodegradability. P90H (Phospholipon®90H, hydrogenated phosphatidylcholine 90%) present a similar structure, characteristics, and applications to DPPC but a lower price in the commercial market.

Wheat has been chosen for Se-biofortification considering its importance as part of society's traditional diet. It is consumed regularly and in considerable amounts by the majority of the population, it is a cost-effective food, and can be enriched with extra nutrients such as Se (Fisinin, Papazyan, & Surai, 2009). Although cereals such as wheat are Se non-accumulators, they may store fairly high Se levels in their tissues without major yield losses, unlike other plants such as legumes (Slekovec & Goessler, 2005).

In this study, the wheat plants were grown in a hydroponic system as a preliminary experiment to avoid interference from possible Se present in the soil, making it the first step before moving on to soil cultivation and large-scale applications (Sambo et al., 2019). The plants were biofortified using a mixture of inorganic selenium salts (sodium selenate and sodium selenite) encapsulated in liposomes. Se uptake, biomass production, concentrations of mineral elements and pigments were analysed. The chemical speciation on Se bioavailability was also studied to better understand the mechanisms of Se absorption and metabolism. Usually, chromatographic analytical techniques coupled with mass spectrometers, such as HPLC-ICP-MS, are used for determining the speciation of Se indirectly. However, several pretreatment steps are required to extract and solubilize the Se species, affecting the structure of the original compounds or producing incomplete recoveries of them (Xie, Sun, Li, Shen, & Fang, 2021). In this work, to perform Se speciation in Se-enriched wheat shoots, X-ray absorption spectroscopy (XAS) measurements were performed considering the intrinsic selectivity of the element and the gentle sample preparation provided by this technique.

2. Materials and methods

2.1. Chemicals

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine 16:0 PC (DPPC) was purchased from Avanti Polar Lipids, Inc. (Alabama, USA) and Phospholipon®90H (P90H, 90% hydrogenated soybean phosphatidylcholine, 4% lysophosphatidylcholine, 2% triglycerides, 2% water, 0.5% ethanol, 1% iodine) was obtained from Lipoid GMBH (Ludwigshafen, Germany). Chloroform (99.2%), 2-morpholinoethanesulphonic acid (MES, ultra-pure grade), potassium nitrate (KNO_3, 99.5%), calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$, 99.7%], mono-ammonium phosphate ($NH_4H_2PO_4$), potassium chloride (KCl), sodium selenite (Na_2SeO_3) and potassium hydroxide (KOH) were procured by VWR International Eurolab S.L.U (Barcelona, Spain). Manganese (II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$), zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$, >99.5%), copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$, >98%), ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), boric acid ($H_3BO_3$, >99.5%), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and ethylenediaminetetraacetic acid iron (III) sodium salt (FeNa-EDTA), seleno-L-methionine (SeMet, >98% TLC), seleno-L-cystine (SeCys, 95%) and Se-(Methyl)selenocysteine hydrochloride (MeSeCys, >95% TLC) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide (H_2O_2) was acquired from Scharlab (Barcelona, Spain), nitric acid (HNO_3, 65%) and sodium selenate (Na_2SeO_4, 98%) from Thermo Fisher Scientific Inc. (Barcelona, Spain), calcium chloride ($CaCl_2$) from Fluka Chemie (Germany). Deionized water was purified through a Millipore purification system from Millipore (Milford, MA, USA). All reagents were used without further purification.

2.2. Liposome preparation

Liposome treatments were prepared using DPPC or P90H by thin-film hydration and further mechanical dispersion following a modified method previously described (Babot-Marquillas et al., 2020). Briefly, DPPC or P90H were dissolved in chloroform at 100 mM. A volume of 2.25 mL of the phospholipids stock solutions was added to a round-bottom flask and diluted with 7 mL of a chloroform/methanol mixture (2:1, *v*/v) with gentle stirring. To obtain a thin film in the flask, the organic mixture was evaporated in a rotary evaporator (Büchi Rotavapor, Switzerland), above the lipids' transition temperatures (Sánchez-Martín et al., 2011). Subsequently, 15 mL of Se solutions prepared as a mixture (1:1, v/v) of selenite and selenate sodium salts in MES buffer

(pH 6.5) were added to the flask to hydrate the film and obtain a dispersion of 15 mM of liposomes using sonication (Branson 2510 Ultrasonic Cleaner, SpectraLab, Canada) in water for 15 min above the phospholipid transition temperature (Tm). A high-intensity ultrasonic probe sonicator (Branson Digital Sonifier 250 & Sonifier Sound Enclosure, Marshall Scientifics LLC, USA) was used to reduce the multilamellar vesicles to unilamellar liposomes with nanometric size. The ultrasonic probe was directly immersed in the samples and the ultrasound was applied. The sonication time and pulses were optimised, considering the initial conditions of Isailović et al. (2013), and finally liposomes were obtained with an amplitude of 40%, pulses on/off about 20 s for 10 min using an ice bath, conditions that result in a homogeneous liposome suspension of <100 nm in size. All liposomal preparations were freshly prepared the day before the application and stored at 4 °C until their use.

2.3. Liposome characterization

After sonication, the size distribution and polydispersity of the liposomes were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS from Malvern Instruments Ltd. (United Kingdom). Each sample was measured three times at room temperature and averages were taken as a result.

cryo-Transmission Electron Microscopy (cryo-TEM, JEOL-JEM-20111, Tokyo, Japan) was used for the visualization of the liposomes obtained as well as for the determination of their morphology in the frozen state. Samples were prepared by depositing 3 μL of liposomal suspension onto a carbon-reinforced holey polymer film covering a copper grid. The deposited sample films were blotted with filter paper, immersed in liquid ethane, and subsequently maintained under liquid nitrogen for the experiment.

2.4. Wheat plants cultures

Seeds of wheat (*Triticum aestivum* L. cv. Bancal) obtained from Fitó S. A. (Spain) were germinated on moistened filter paper for 5 days at 25 ± 1 °C in the dark and 2 days in the light. Homogeneous seedlings were grown hydroponically (12 plants per 6 L pot) in a continuously aerated ½ strength Hoagland's nutrient solution, in a controlled environment under the following conditions: 16 h day/8 h night photoperiod with a light intensity of 320 $\mu E \cdot m^{-2} \cdot s^{-1}$. The composition of Hoagland's solution was: 3 mM of KNO_3, 2 mM of $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM of $NH_4H_2PO_4$, 0.5 mM of $MgSO_4 \cdot 7H_2O$, 1 μM of KCl, 25 μM H_3BO_3, 2 μM $MnCl_2 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $CuSO_4 \cdot 5H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$,) and 20 μM FeNa-EDTA. The pH of the solution was buffered with 2 mM MES and adjusted to 6.5 with 2 M KOH.

2.5. Selenium treatments application

Selenium biofortification treatments, with different total Se concentrations of mixture of selenite and selenate (100, 500 and 1000 μM), with and without liposomes were applied twice by spraying the different solutions on the leaves of the plants in the 5th and 7th week of the plant growth cycle. The treatment without liposomes will be referred to as Se100, Se500 and Se1000, respectively, and when Se is encapsulated in DPPC liposomes as Se100_D, Se500_D and Se1000_D. In the case of P90H, only the 500 μM treatment was applied to compare it with DPPC (Se500_P). A control experiment was also performed without any treatment (Control). All treatments were applied to plants in two pots of the hydroponic culture. One week after each application, 4 plants per pot were harvested for further analysis. After harvesting, roots were washed with ice-cold 2 mM of $CaCl_2$ to remove remaining surface nutrients. The plants were divided into roots and shoots and fresh weights (FW) were determined. Dry weights (DW) were measured after lyophilization. All samples were ground and stored at room temperature for analysis.

2.6. Chlorophylls and carotenoids determination

The pigments were determined using Eq. (3) and Eq. (4) as total chlorophylls (T_{Chl}) and total carotenoids (T_{Car}), respectively. The extraction of pigments was done with acetone:water (80:20, *v*/v). For this, 100 mg of lyophilized and ground shoots was used. The absorbance of the extracts was measured at 440, 646 and 663 nm (UV double-beam spectrophotometer, UNICAM, model UV2-200, USA). The pigment concentration was determined using the chlorophylls equations, Eqs. (1) and (2) (Lichtenthaler & Wellburn, 1983), and carotenoids equation (Holm, 1954) where A is the absorbance of the samples at the corresponding wavelength (λ). The results were expressed as mg pigment/100 g DW.

$$Chl_A\ (\mu g \cdot mL^{-1}) = 12.21 \cdot A_{663} - 2.81 \cdot A_{646} \quad (1)$$

$$Chl_B (\mu g \cdot mL^{-1}) = 20.13 \cdot A_{646} - 2.81 \cdot A_{663} \quad (2)$$

$$T_{Chl}\ (\mu g \cdot mL^{-1}) = Chl_A + Chl_B \quad (3)$$

$$T_{Car} (\mu g \cdot mL^{-1}) = 4.69 \cdot A_{440} - 0.268 \cdot T_{Chl} \quad (4)$$

2.7. Determination of total selenium, macro and micronutrients concentration

Total concentrations of Se, micro (B, Mn, Fe, Cu, Zn, Mo) and macronutrients (Mg, S, P, K, Ca) concentrations were analysed by Inducted Coupled Plasma-Mass Spectrometry (ICP-MS). Prior to analysis, the samples were acid digested (shoots and roots) with 7 mL of 65% nitric acid and 3 mL of H_2O_2. The digestions were carried out in an analytical microwave (Mars 5, CEM, USA) with a temperature and pressure gradient up to 180 °C and 1.9 atm for 45 min.

After digestions, the samples were filtrated and diluted and the concentrations of ^{11}B, ^{55}Mn, ^{56}Fe, ^{64}Zn, ^{65}Cu, ^{78}Se, ^{98}Mo, ^{24}Mg, ^{31}P, ^{39}K and ^{44}Ca were measured by ICP-MS (X Series 2, Thermo Elemental, UK). The macronutrient S was measured by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Agilent 5900, USA).

2.8. Selenium speciation analysis by XAS

X-ray absorption near edge structure (XANES) spectra were collected at Se K-edge in CLAESS beamline at ALBA Synchrotron, Barcelona, Spain (Simonelli et al., 2016).

Approximately 30 mg of lyophilized and ground wheat shoots, from plants biofortified with Se after two applications of the treatments, were homogenized, pressed into pellets (5 mm diameter). Each sample was prepared by mixing 3 different replicates.

The measurements were performed in fluorescence mode due to the low concentration of Se in the plant samples using the multi-element Si drift detector with XSpress3electronics. The spectra were collected on 3 spots of each pellet to account for the sample variability and possible inhomogeneities. All the measurements were performed at liquid nitrogen temperature to avoid radiation damage of the samples.

Furthermore, reference samples of sodium selenite, sodium selenate, seleno-L-methionine, seleno-L-cystine and Se-(Methyl)selenocysteine hydrochloride were measured for comparison. The references were prepared as pellets and as aqueous solutions for comparison with the samples considering the possible changes between the structure of the compounds in solid state and in the plants. The solutions were loaded into an in-house designed liquid cell with a 2 mm transmission path and Kapton windows (Marini et al., 2018). The references were measured in transmission mode. For energy calibration, the Se K-edge XANES spectrum of the elemental selenium was used and the energy of the maximum of the first derivative was set to 12658 eV.

Normalization of the XANES data using standard procedures and linear combination fitting analysis were performed with the ATHENA

program of the DEMETER software package (Ravel & Newville, 2005).

2.9. Statistical analysis

The statistical analysis of the results was done by one-way analysis of variance (Anova) with OriginPro 9.0 software to determine the differences between control and Se treatments. The comparison of the means was done by the Tukey test. All evaluations were done at a 5% level of significance ($p\text{-value} < 0.05$). Statistically significant differences are indicated with different letters in the charts.

3. Results

3.1. Liposomes characterization

Fig. 1 shows cryo-TEM images of Se500_D (DPPC) and Se500_P (P90H) (Fig. 1A and B, respectively) liposomes and their size distribution by DLS (Fig. 1C and D, respectively). The results indicate that the vesicles present homogeneous size distributions with average sizes of 28.87 and 65.76 nm and polydispersity index (PdI) was lower than 0.3 in both cases, 0.196 and 0.247, respectively. The P90H vesicles showed a larger distribution of the liposomes size (Fig. 1D) than DPPC liposomes (Fig. 1C), may be related to the lower purity of P90H compared to DPPC and the presence of other compounds in the formulation and the higher Tm. However, both liposomes show average particle sizes below 100 nm after the ultrasonication, which will allow them to be easily absorbed through the plant leaves.

The cryo-TEM images revealed characteristic spherical structures of liposomes of unilamellar vesicles for both DPPC and P90H liposomes loaded with Se (red and green circles in Fig. 1A and B). Furthermore, both samples contained polyhedral shapes such as hexagons and disc-like shapes (white circles) formed due to the rupture of the vesicles during the preparation steps for cryo-TEM analysis at temperatures below Tm (Ickenstein, Arfvidsson, Needham, Mayer, & Edwards, 2003; Meister & Blume, 2017).

3.2. Growth features of enriched wheat plants

Foliar application of selenium loaded in liposomes may have positive or negative effects on plants at the physiological, biochemical, and molecular levels. This impact is determined by physical and chemical properties of liposomes, wheat characteristics, Se concentration, and others. Foliar application of treatments can promote growth, biomass production, and yield in some agricultural crops, but can also cause nutrient deficiency, retard root elongation growth, and delay flowering in others (Avellan et al., 2021). In order to evaluate the impact of Se-loaded liposomes on wheat plant physiology and biochemistry, the plant biomass, and the concentration of pigments (chlorophylls and carotenoids) in comparison to the control conditions, were analysed. The DW of shoots and roots of the treated wheat plants and the pigments concentration of the shoots were analysed after the two harvests (Table 1). These physiological parameters were compared among the different treatments applied to evaluate their effect on wheat development and yield. For both, shoots and roots, there is a significant increment in the total DW after the second application regarding the first one, indicating that after two applications of the foliar treatments the plant growth process was not affected. In addition, no significant differences were observed among treatments and compared to the control in the different applications.

The concentration of chlorophylls (Table 1) showed no significant differences between the different treatments and applications. The level of carotenoids was also similar between treatments but after the second application there was a significant increase in these compounds compared to the first application.

The results suggest that the different concentrations of Se applied with or without liposomes do not affect their growth and pigments concentration. In wheat and other cereals, growth reduction, decrease of photosynthetic activity, chlorophylls, and carotenoids are related to Se

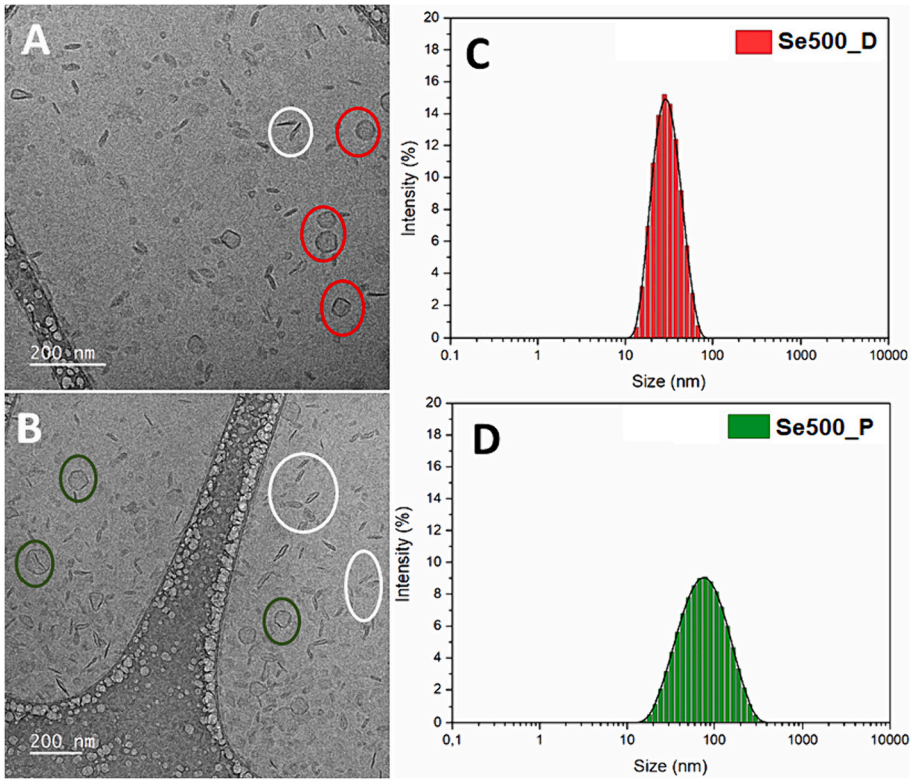

Fig. 1. *Cryo*-TEM images using 20kx of magnification of DPPC (Se500_D) (A) and P90H (Se500_P) (B) liposomes loaded with selenium, size distribution by intensity measured using dynamic light scattering with average particle size of 28.87 for Se500_D and 65.76 nm for Se500_P (C and D, respectively).

Table 1
Growth parameters (dry biomass, total chlorophylls, and carotenoids) of wheat plants biofortified with different Se treatments after one or two applications.

Treatment	Dry biomass (g DW)				Total Chlorophylls (mg/ 100 g DW)		Total Carotenoids (mg/ 100 g DW)	
	Roots		Shoots					
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Control	1.32 (5) ^{a}	1.94 (3) ^{b}	4.14 (8) ^{c}	5.9 (5) ^{d}	300 (14) ^{e}	321 (7) ^{e}	13 (2) ^{f}	37 (1) ^{g}
Se100	1.20 (7) ^{a}	2.56 (1) ^{b}	3.3 (2) ^{c}	5.1 (2) ^{d}	350 (25) ^{e}	272 (2) ^{e}	18 (2) ^{f}	34 (4) ^{g}
Se100_D	1.30 (1) ^{a}	2.58 (1) ^{b}	4.2 (1) ^{c}	5.0 (1) ^{d}	263 (12) ^{e}	313 (3) ^{e}	14 (6) ^{f}	41 (1) ^{g}
Se500	1.42 (2) ^{a}	3.11 (3) ^{b}	3.6 (3) ^{c}	6.1 (6) ^{d}	303 (14) ^{e}	367 (4) ^{e}	21 (6) ^{f}	42 (5) ^{g}
Se500_D	1.32 (1) ^{a}	2.57 (2) ^{b}	3.8 (2) ^{c}	5.3 (4) ^{d}	341 (15) ^{e}	260 (4) ^{e}	23 (6) ^{f}	36 (6) ^{g}
Se500_P	1.40 (3) ^{a}	2.62 (2) ^{b}	3.5 (5) ^{c}	5.16 (9) ^{d}	314 (21) ^{e}	331 (2) ^{e}	21 (5) ^{f}	51 (4) ^{g}
Se1000	1.55 (2) ^{a}	2.63 (2) ^{b}	3.7 (2) ^{c}	4.9 (3) ^{d}	330 (33) ^{e}	320 (2) ^{e}	20 (4) ^{f}	41 (2) ^{g}
Se1000_D	1.43 (5) ^{a}	2.22 (9) ^{b}	4.0 (2) ^{c}	6.2 (5) ^{d}	334 (35) ^{e}	361 (6) ^{e}	25 (1) ^{f}	44 (1) ^{g}

Note: Results are expressed as means ± SE ($n = 5$). Letters represent significant differences among groups (Tukey test, p-value < 0.05).

phytotoxicity due to the modification of biochemical and physiological process but these negative effects are directly related to the Se concentration and species, exposure time, plant species and cultivation stage at the time of Se application (Hasanuzzaman et al., 2020; Sharma, Kaur, Kaur, & Nayyar, 2017; Wang et al., 2020). Our results suggest that the amount of pigments is variable throughout the cultivation, increasing during leaf development and not due to the Se application, but probably due to the level of Se reached in the plants. Furthermore, no morphological or physiological changes were observed in the plants after biofortification.

3.3. Total Se concentration in wheat

The present work focuses on the possibility of using liposomes to improve Se uptake in foliar agronomic biofortification. Even though the edible part of the wheat is the grain, the shoots are also used as animal fodder. For this reason, the plants were harvested immediately after the application of the treatments (one week later) and the analysis were realized in shoots and roots with emphasis on the shoots (stems and leaves) where the treatments were applied.

Fig. 2 illustrates that the total Se increases significantly from the first to the second application. In addition, as expected since the application was made foliarly, the shoots accumulated more Se than the roots. For the first application, there are no significant differences between Se100, Se100_D and Se500 treatments and the control. Therefore, no notable amount of Se was accumulated with these treatments. In the case of Se500_D and Se500_P treatments, the concentration levels are significantly higher than those of Se500. These differences between treatments with and without liposomes are greater for the highest concentration considered, Se1000 and Se 1000_D, confirming the previous observation.

After two applications, Se500 treatment is significantly higher than Se100 and Se100_D, and Se500_D and Se500_P accumulated more Se than Se500. The same thing happens between Se1000_D and Se1000. Overall, Se1000_D treatment is the one that accumulates the most selenium in both cases (first and second application). In the roots there were no differences in the accumulation of Se between applications. Wheat plants treated once with Se500_P showed the same Se concentrations than those treated with the double concentration of Se without liposomes (Se1000). Depending on the treatments, the selenium uptake ranged from 7 to 17% of the initially given Se considering that a volume of 7.5 mL was applied each time (Table 1 in SI file). However, more studies are required to obtain more results and a definitive conclusion on the effect of the different phospholipids, since only one concentration was tested for P90H.

These results suggest that liposomes are effective as Se nanocarriers in the wheat foliar biofortification, as the uptake of Se increases approximately 1.5-fold when liposomes are used.

In general, plants take up nanocarriers applied to the foliage, usually through stomata and trichomes. Stomata penetration may be the main absorption pathway on the leaf surface since absorption through the epidermis is very limited due to pore size of this structures. The foliar absorption of nanoparticles may subsequently be followed by processes organized at the cellular level, which relate to the interaction between

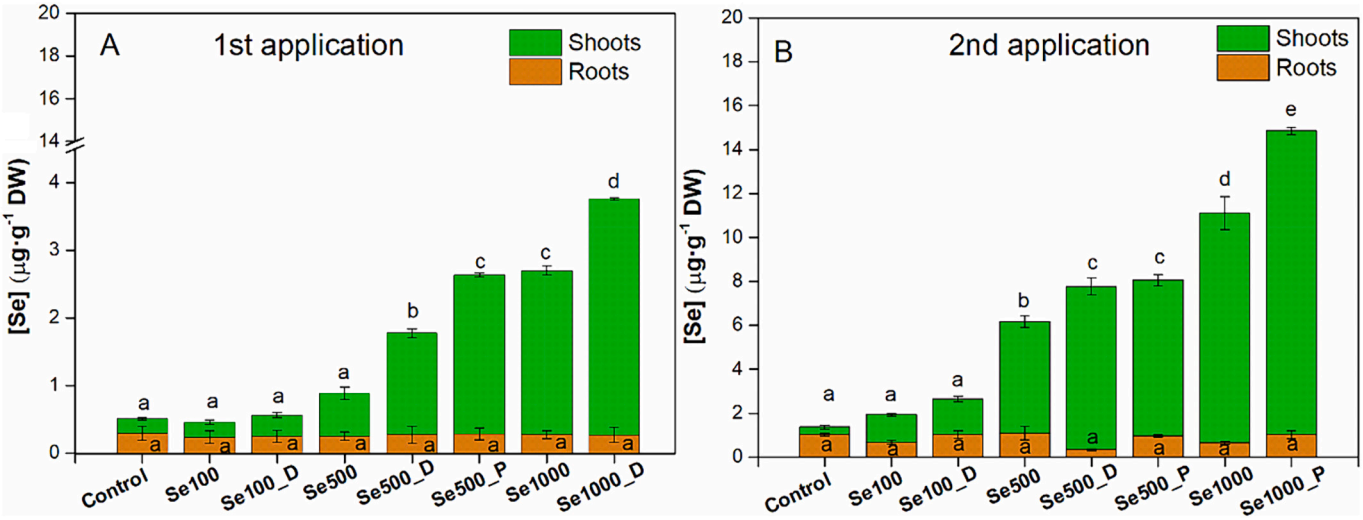

Fig. 2. Selenium concentration in roots and shoots (μg Se·g^{-1} DW). The results obtained from the first (A) and the second (B) application of the treatments are expressed as means ± SE ($n = 4$). Different letters represent significant differences among groups of shoots or roots (Tukey test, *p-value < 0.05*).

nanoparticles and specific cellular barriers such as cell walls, plasma membrane and/or organelle membranes like endocytosis or transport through membrane-bound proteins (Hong et al., 2021). Previous work suggests that liposomes can penetrate plant leaves possibly thought the stomata with sufficiently large pore size and release their cargo into the cytoplasm or nuclei of plant cells in a 72 h period, where the disruption of the liposomal membrane appears to be due to endogenous factors (*e.g.* cytoplasmic lipases) and osmotic destabilization with posterior accumulation in the vacuoles cells, without causing toxicity to plants, probably due to their biocompatibility. The loaded liposomes can be translocated throughout the plant using the plant natural transport mechanisms with the possibility of reaching the grains and without exerting any toxicity (Karny et al., 2018). In our work, there was not a significant translocation of Se from leaves to roots (Fig. 2) indicating that there was no accumulation of Se in this organ, which could cause toxic effects. Selenium concentrations determined in the shoots of wheat plants are a promising result for foliar applications with liposomes, especially for the phospholipid P90H because it can play an important role in agriculture, being more cost-effective than DPPC with similar biofortification results.

3.4. Mineral elements uptake

The interaction among mineral elements in plants is well-known and affects the nutrient status of plants. The concentration of macro (S, Mg, K, Ca, and P) and micronutrients (Fe, Mo, Mn, B, Cu, Zn) was evaluated in shoots and roots of Se-treated wheat plants after each application. The influence of Se on nutrients did not follow a general trend, but individual behaviour can be observed for different elements.

The analysis of the total concentration of macronutrients (Fig. 3 for S and Fig. S1 for Mg, P, K and Ca) revealed that the concentration of S increased in both plant tissues (roots and shoots) between applications and significant differences were only observed after the first application between treatments and control. Similar level of Mg and K in shoots and roots after each application indicate a relatively consistent ability of wheat to accumulate these macronutrients considering their importance for plant development. In addition, Ca increased in the shoots (~ 40%) from the first to the second application for all treatments, and similar values were obtained for the roots. In the case of P, the concentration decreased in both shoots and roots after the second application.

Micronutrients are essential for enzymatic reactions, protein synthesis and affect the plant antioxidant behaviour. As shown in Fig. 3C-D and Fig. S2, the accumulation of micronutrients shows more variable results. Molybdenum (Fig. 3C-D), and Fe and Mn (Fig. S2) showed notable differences since, after the second application, the total amount of these nutrients decreased in the plant tissues. Boron, Zn and Cu (Fig. S2) maintained the same level after both applications of the treatments with small differences only in the levels of B and Zn in the

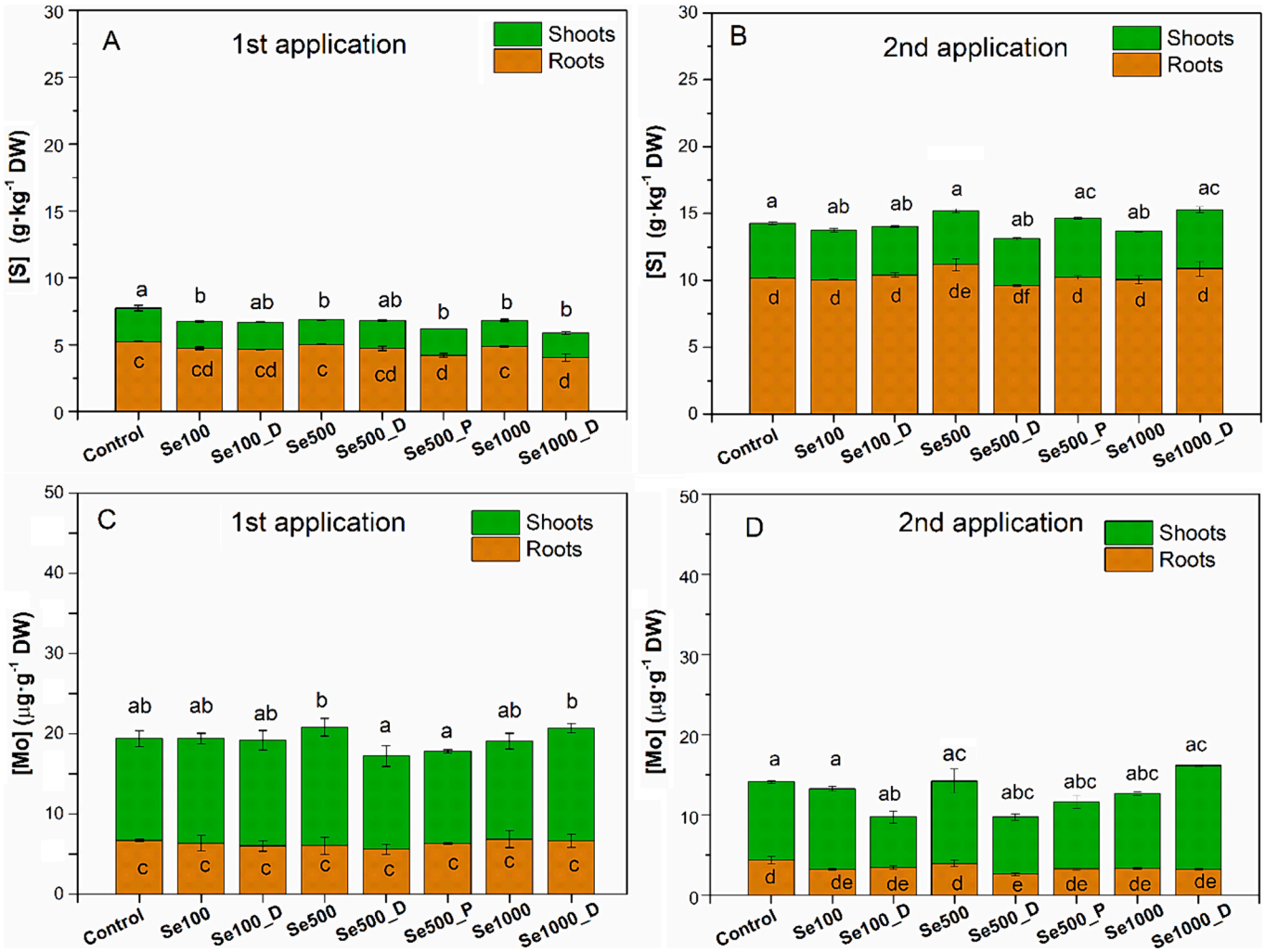

Fig. 3. Sulphur (A, B)(g S·kg^{-1} DW) and molybdenum (C, D) (μg Mo·g^{-1} DW) concentration in roots and shoots after the first and the second application of the treatments. The results obtained are expressed as means ± SE ($n = 5$). Different letters represent significant differences among groups of shoots or roots (Tukey test, *p-value* < *0.05*).

roots. Significant differences were also observed for Mn in shoots and in the three (Mn, Zn and B) in roots between treatments.

In summary, no synergistic or antagonistic interactions can be established in the influence of Se-liposome treatments at different concentrations on the uptake of mineral nutrients by wheat plants. However, in some cases significant changes were observed between applications and total nutrient accumulation. The most relevant effects were the increase in S and Ca concentrations in shoots, the decrease of P and K in roots, and the decrease of Mo, Mn and Fe in both tissues. Selenium species are taken up, translocated, and metabolized within the plant by the S and P transporters and metabolic pathways (White, 2018). In this study, since the Se uptake occurs thought the leaves and the uptake of minerals thought the roots, it is expected that the most important modifications in the mineral state of plants occur in the process of transport, translocation and accumulation of S and P. However, no shared metabolic pathways are known between Se and the other nutrients. Therefore, the reason for the modification of their content in the plant may not be due to Se uptake and translocation processes or the presence of liposomes, but rather to indirect interactions, induced toxicity, and alteration of the plant homeostasis. Selenium ions can affect the transport of other ions in the plant by changing the permeability coefficient of plasma membranes for some species. Nevertheless, the concentrations of nutrients are at an adequate level for the development of wheat plants, which determines their health and subsequent consumption, as reported in other works (Nawaz, Ahmad, Ashraf, Waraich, & Khan, 2015; Souza et al., 2014).

3.5. Selenium speciation by XAS

XAS allows a direct determination of the Se species without having to digest the plant sample with chemical treatments or having to isolate the chemical species using extraction processes as it is neccesary when using indirect speciation techniques such as HPLC-ICP-MS. Se K-edge XANES spectra of the standards (inorganic and organic Se species) and wheat shoots samples are shown in Fig. 4. The spectra of the Se references showed a prominent feature at the beginning of the spectra (called white-line) which appears at different energy positions depending on the different chemical state atomic the selenium atom in the chemical compound. For the organic references containing C-Se-C bond (*i.e.*, SeMet and MeSeCys) the spectra are almost overlapped and display a narrow white-line having its maximum at 12661 eV. However, the SeCys reference, which consist of a C-Se-Se-C bond, presents a white-line at slightly lower energy, 12660 eV. The inorganic selenium species showed a more intense white-line with maxima at 12667 eV and 12664 eV for selenate (SeVI) and selenite (SeIV), respectively.

The XANES spectra of the wheat samples (Fig. 4B-D) showed two main spectral features that can be attributed to the presence of organic Se and inorganic selenate by direct comparison with the Se standards. When comparing the spectra of the different treatments, the relative intensity of these two features for the samples with liposome treatments change respect to those samples without liposomes. There is a slight increase of the intensity feature related to the organic species and a decrease of the feature atributted to the selenate contribution. This has been found for all the Se concentrations analysed, corroborating that there is a change in the contribution of the different Se species to the final spectra. To get a further insight, the linear combination fitting (LCF) analysis of the sample spectra using the Se standards was performed. This provides a semi-quantitative analysis of the amount of each Se species present in the shoots (Table 2).

The results from the LCF analysis confirmed that after the application of the inorganic Se treatments to the plants, either in presence or absence of liposomes, Se is mainly present as organic Se in the form of seleno-amino acids (SeMet, MeSeCys and SeCys). This agrees with previous works on Se speciation analysis of wheat plants biofortified with inorganic Se species in hydroponic system (Di et al., 2023; Subirana, Boada, Xiao, Llugany, & Valiente, 2023; Xiao, Boada, Marini, Llugany, &

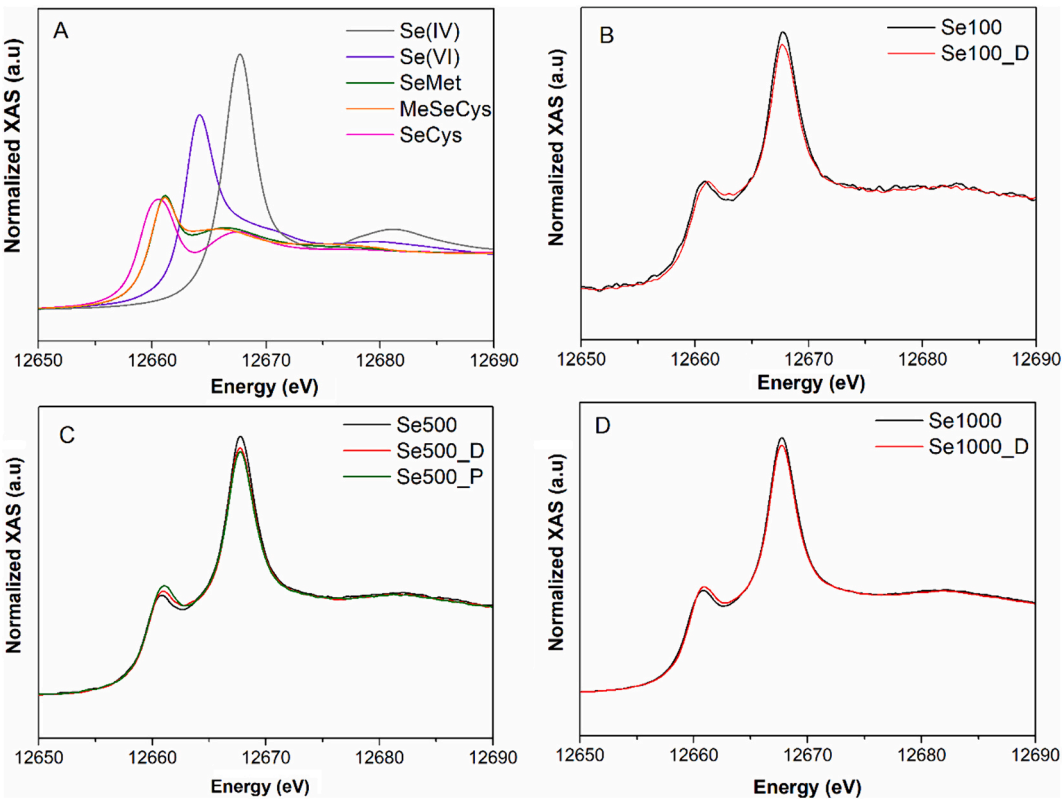

Fig. 4. Selenium K-edge XANES spectra of: (A) different selenium references, (B) wheat shoots biofortified with Se 100 μM with and without liposomes, (C) Se 500 μM with and without liposomes, (D) Se 1000 μM with and without liposomes (a.u., arbitrary units).

Table 2
Results from LCF analysis of Se K-edge X-ray absorption spectra collected on samples.

Treatment	R-factor	Relative concentration (%)			
		C-Se-C	C-Se-Se-C	Se(VI)	Se(IV)
Se100	0.0074	28.9	46.7	17.8	n.d
Se100_D	0.0061	48.0	28.8	15.9	n.d
Se500	0.0078	32.2	39.2	18	n.d
Se500_D	0.0069	40.7	33.3	16.5	n.d
Se500_P	0.0061	41.8	34.3	15.8	n.d
Se1000	0.0082	29.4	43.3	17.4	n.d
Se1000_D	0.0074	38.8	35.9	16.3	n.d

Notes: R-factor is a measure of the mean square sum of the misfit at each data point which indicates the goodness of fit.
C-Se-C refers to components with a C-Se-C bond structure (i.e., SeMet and MeSetCys), and C-Se-Se-C to C-Se-Se-C bond structure (i.e., SeCys).

Valiente, 2020). In addition, it was possible to confirm that the samples with liposomes had an increase of 2.6% aproximately of the total organic Se content at expenses of the inorganic selenate.

Since an equimolar mixture of Se(IV) and Se(VI) was applied in the treatments and that the speciation results indicate that the presence of Se(IV) in the wheat shoots is negligible, it is posible that almost all the concentration of this inorganic species was transformed into organic Se by the plants. This process occurs with the assistance of O-Acetyl serine complexes, Seleno methyl transferase, Cysteine reductase and other Se reducing enzymes as has been reported before (Ellis & Salt, 2003). Normally, this biochemical rute is less efficient for Se(VI) than from Se (IV), since Se(VI) needs a prior reduction to Se(IV) (Schiavon et al., 2020). Indeed, this is consistent with the remaining amount of Se(VI) that can be observed in the present work.

The results from the LCF analysis revealed that in Se-liposomes treatments, selenium predominantly exists as Se-amino acids with a C-Se-C bond, whereas in Se treatments without nanocarriers, the predominant form is C-Se-Se-C, this is as SeCys. This difference is evident across all three studied Se concentrations and for both DPPC and P90H liposomes. Considering the metabolic pathway of selenium in plants, when a higher proportion of Se(IV) is available, the plants are able to convert Se more easily into SeMet or MeSeCys species, passing through SeCys (El-Ramady et al., 2015; Wang et al., 2020). Thus, the increase in the absorption of Se(IV) due to the applications with liposomes produces an increase in the C-Se-C forms. As selenate can be stored in leaf vacuoles in a way that it is harmless to the plant (Subirana et al., 2023), and considering that the C–Se–C species are less harmful for plants since the incorporation of SeCys into protein could interfere with the formation of disulfide bridge affecting tertiary structure of S-proteins (Terry, Zayed, De Souza, & Tarun, 2000), our results show that the use of liposomes for foliar applications not only increases the total Se uptake but also decreases the possible toxic effects of the Se organic species to the plants.

From the perspective of human nutrition and health, two aspects should be considered: the concentration of Se in the edible parts of wheat (grains) and the specific chemical forms of Se obtained after plant metabolism, since different forms of Se have different nutritional value and toxicological properties in the human body (Zhou, Yang, Kronzucker, & Shi, 2020). For example, MeSeCys is relatively non-toxic to cells, and it is considered one of the most promising antineoplastic agents (Gandin, Khalkar, Braude, & Fernandes, 2018). SeMet is one of the major dietary sources of Se, but it is not in a biologically active form. It can be transformed to selenocysteine or be unspecifically incorporated into proteins due to its similarity to methionine, and stored until the proteins are degraded, at which point then it can be recycled (Weekley & Harris, 2013). SeCys can be reduced to highly reactive selenolate by thioredoxin reductase or excess cysteine and glutathione. When SeCys is present at high concentrations, selenolate-mediated biochemical reactions disrupt cellular redox homeostasis and induce oxidative stress. Limited pharmacokinetic studies suggest that SeCys is well tolerated in mice and rats, with a narrow window between no observed effects and toxicity. SeCys also exhibits antitumor activity with high selectivity between cancer and normal human cells by inducing an increase in ROS in selected cancer cell lines (Chen & Wong, 2009; Misra & Björnstedt, 2018).

Se accumulation in grains after Se biofortification using liposomes may be the next step in future work to meet the Recommended Daily Allowance (RDA) of Se at least partially for adults, which is set at 55 μg $Se{\cdot}day^{-1}$ (Institute of Medicine, Food and Nutrition Board, 2000).

4. Conclusions

Our results show that Se loaded DPPC and P90H liposomes were successfully prepared and applied by foliar feeding to wheat plants grown in hydroponic medium. The different Se treatments (with or without liposomes) did not affect plant growth or pigment accumulation, indicating that the plants successfully absorbed Se. In addition, the Se liposome treatments did not affect the uptake of mineral nutrients by the plants. P90H liposomes can be considered a better alternative than DPPC for Se biofortification of wheat plants, since similar results were obtained with both and the price of P90H phospholipid is considerably lower. Speciation analysis of the XANES spectra revealed that Se in wheat is mainly present as organic Se and that foliar application using liposomes influences the proportion of organic Se species, with SeMet and MeSeCys predominant over SeCys. In view of the results obtained, new experiments will be carried out until the development of the grain and full maturity of the plants to analyze the accumulation of Se in them. More studies should be conducted in soil and with a larger number of plants to investigate the scalability of the process in a controlled environment in the initial stages. On the other hand, the economic viability and scalability of using Se-loaded liposomes for biofortification should be evaluated: production and application costs, and its comparison with other biofortification strategies. Also, a market analysis will be needed in order to assess the biofortified product demand in the market or the consumer preferences. But, in conclusion, agronomic biofortification with liposomes may be a successful method to increase Se and other nutrients supplied to plants without contamination and to produce foods enriched in organic Se species that promote human health.

Authors contribution

Marcia Viltres Portales contributed to the experimental design and setup, data collection and analysis, first manuscript draft writing and discussion of the results. María Jesús Sánchez Martín contributed to project conception and experimental design, supervision, discussion of the results and manuscript writing. Roberto Boada contributed to experimental design, XAS data collection and interpretation, discussion of the results and manuscript revision. Mercè Llugany contributed to the experimental design, data interpretation, discussion of the results and manuscript revision. Manuel Valiente contributed to the project conception, funding, supervision, discussion of the results and manuscript revision. All authors approved the manuscript.

CRediT authorship contribution statement

Marcia Viltres-Portales: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **María-Jesús Sánchez-Martín:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Roberto Boada:** Writing – review & editing, Software, Formal analysis, Data curation. **Mercè Llugany:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Manuel Valiente:** Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139123.

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