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Accumulation and speciation of selenium in biofortified vegetables grown under high boron and saline field conditions

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ABSTRACT

Selenium (Se) biofortification, as an agronomic-based strategy, is utilized to produce Se-enriched food products for increasing Se intake in inhabitants in Se-deficient regions. This strategy can be accomplished by soil and foliar application of Se or by growing crops in soils naturally high in Se. In this study, different cruciferous vegetables were field-grown in high boron (B) and saline soils of central California containing naturally high levels of Se. We investigated whether Se biofortification occurs in salt- and B-tolerant vegetables grown in poor-quality soil. The uptake of Se and other elements occurred in all vegetables. In plant tissues, Se speciation analyses showed greatest percentages of Se-containing compounds were contained in organic Se forms (monomethylated) and as selenate in the inorganic Se forms. Selenium-enriched vegetables produced from saline soils high in B and Se can be a natural source of Se-biofortified food that can be consumed as bioactive food products.

1. Introduction

Plant foods are the major dietary sources of Se in many countries around the world, followed by meats and seafood (Ods, 2016). Human Se intake and Se status in a population depends firstly on Se concentration in soils, and the resulting Se concentrations in the harvested edible plant product grown in these soils. This soil to plant Se relationship will determine if an area is a Se-deficient or Se-toxic region (Dos Reis, El-Ramady, Santos, Gratão, & Schomburg, 2017). In a Se-deficient region, one of the most promising approaches to mitigate a low transfer of Se from soil into the food chain involves the use of a strategy called Se biofortification (Bañuelos, Lin, & Broadley, 2017; Schiavon & Pilon-Smits, 2017; White & Broadley, 2009). Selenium biofortification, as an agronomy-based strategy, can be utilized to produce Se-enriched food products that may help reduce dietary deficiencies of Se occurring throughout susceptible regions of the world (Broadley et al., 2006). Excellent review articles have described other successful biofortification strategies for reducing micronutrient deficiencies (Bouis & Welch, 2010; Carvalho & Vasconcelos, 2013; Saltzman et al., 2013). In addition, a large biofortification platform—The Harvest Plus Challenge Program—was created in 2004 for reducing malnutrition in Asia and Africa (Carvalho & Vasconcelos, 2013). Before we can effectively develop a Se-biofortification strategy, it is important to understand that there is no conclusive evidence demonstrating Se as an essential nutrient required by typical agronomic plants (Pilon-Smits, 2015). Hence, selenate, as the most common form of Se taken up by the plant, is transported throughout the plant via the sulfate transport system (Guignardi & Schiavon, 2017; Sors, Ellis, & Salt, 2005). Selenium bioavailability and its transfer into the food chain will depend on its speciation in the soil environment, which is mainly influenced by the prevailing pH, redox potential, organic matter content, and soil age, and is mediated through chemical and biological Se transformations and interactions with microorganisms and plants (Patel, Trivedi, Shah, & Saraf, 2018; Li et al., 2017). Moreover, the similar chemical and physical properties between Se and sulfur (S) are essential for the success of Se biofortification strategies. In this regard, Se can be commercially added as an inorganic fertilizer to Se-deficient soils, e.g., in Finland (Alfthan et al., 2011), and absorbed via S pathways into the plants. The application of inorganic Se sources to field soils supporting cropping systems has been studied worldwide, e.g., United Kingdom (UK) (Hartikainen, 2005; Lyons, 2010; Stroud et al., 2010), Europe (Poblaciones, Rodrigo, Santamaría, Chen, & Mcgrath, 2014), New Zealand (Curtin, Hanson, Lindley, & Butler, 2006), Africa (Chilimba et al., 2012) and China (Dinh et al., 2018; Wu et al., 2015), as well as the foliar application of Se (Smrkolj, Stibilj, Kreft, & Germ, 2006; Kápolna, Laursen, Husted, & Larsen, 2012) and the application of Se-enriched plant material to soil as a green fertilizer (Bañuelos, Arroyo, Pickering, Yang, & Freeman, 2015; Wan, Zhang, & Adhikari,

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2018) To better understand the relationship between Se in the environment (including total Se in soil, water, plants, and food) and daily Se intake, Dinh et al. (2018) reviewed the Se distribution in the environment, plant uptake of Se, and its effects on human health in China.

Another Se biofortification option is to exploit the possibility of producing Se-enriched food and feed products from plants grown in soils naturally abundant in Se (natural biofortification), like those soils found in Enshi and Ziyang, China (Dinh et al., 2018); South Dakota, USA (Gerla, Sharif, & Korom, 2011), and Punjab, India (Dhillon & Dhillon, 2009). In China, Brazil, and California, food products grown in different Se-rich soils have produced food products with higher Se concentrations (Da Silva, Mataveli, & Arruda, 2013; Bañuelos et al., 2015; Dinh et al., 2018). Similarly, Bañuelos (2002) reported on producing Se-enriched broccoli when irrigated with water containing naturally occurring Se. Identifying areas with naturally high levels of Se in soil and water and evaluating processes controlling soil Se distribution is, however, key for producing crops that can be naturally biofortified with Se (Jones & Winkel, 2017). Although weathering and transport of Se from soil deposits are natural processes, the release of excessive levels of geogenically derived Se into the environment can additionally be triggered by human actions, or by dusts released in the vicinity of coal burning sites, while industrial and agricultural activities are the dominant anthropogenic sources of Se pollution (He et al., 2018). The classical example of how agricultural activities can accelerate the solubilization, movement, and accumulation of naturally occurring Se in the ecosystem was observed in aquatic organisms and birds frequenting the Kesterson reservoir (San Joaquin Valley, California) in the late 1980s.

For natural biofortification strategies, understanding the impact that chemical processes have on solubility and mobility of Se in the environment is key (Fernández-Martínez & Charlet, 2009). In the environment, Se can exist in the (-II), (0), (IV), and (VI) oxidation states, and in general, Se solubility, and therefore mobility, increases with increasing redox potential (i.e., more oxidizing conditions). Depending on the soil type among the different fractions present in the natural soil, selenate and selenite are generally most bioavailable to plants (Pilon-Smits, 2015). Overall, Se bioavailability to plants under large-scale conditions is poorly characterized due to the lack of studies that quantify the collective and variable factors known to affect Se mobility and uptake in the field environment. Most studies have been conducted in controlled environments with uniform soil Se concentrations (e.g., greenhouse pot studies), which oversimplifies the chemical, physical, and biological processes and variable soil Se levels present in natural field growing conditions. Growing crops in soils naturally high in Se is of great interest worldwide, especially in China, where over 500 million people experience some form of Se deficiency (Dinh et al., 2018). Selenium-biofortified food crops will contain Se predominately as organic Se compounds and secondarily as inorganic compounds. Consumption of Se-biofortified plant products containing organic Se forms may lead to a higher intake of Se. Moreover, organic forms of Se such as mono methylated selenoamino acids, e.g., methylselenocysteine, gamma-glutamyl-methyl-selenocysteine contained in some Se-enriched plant tissues, may be more readily used by enzymes for promoting antioxidant activities in humans. In this regard, others have reported that organic Se compounds contained within wild onions (Ramps) (Whanger, Ip, Polan, Uden, & Welbaum, 2000), garlic (Ip & Ganther, 1992), and broccoli (Finley et al., 2001), may also be involved with carcinogen defense activities. Hence, speciation of Se compounds in Se-biofortified vegetables produced via natural biofortification strategies is important in determining the potential health benefits related to increased Se intake and absorption and potential relationships to antioxidant enzymatic activities.

In this study, we investigated the ability of selected salt- and boron (B)-tolerant cruciferous vegetables, as sulfur-loving plants, to accumulate Se when field-grown in high saline-B field soils containing high and variable levels of Se and other natural elements. We hypothesize that we can measure an accumulation of Se in these vegetables and quantitatively and chemically speciate the different forms of Se in the plant tissue, as well as identify the potentially higher accumulation of other naturally occurring essential micro- and macronutrients in the vegetable tissues.

2. Methods and materials

Brassica species broccoli (*Brassica oleracea* var. *italic*), red cabbage (*Brassica oleracea* var. *capitata f. rubra*), green cabbage (*Brassica oleracea L. var capitata*), and savoy cabbage (*Brassica oleracea* var. *sabauda L.*) along with Swiss chard (*Beta vulgaris L. var cicla*) were initially started from seed in seedling trays under greenhouse growing conditions and field transplanted as 3-week-old plants into the field site (described below) at Five Points, CA in the west side of the San Joaquin Valley. (Note: although the selected cruciferous vegetables are considered cool season crops, we, however, planted them in late spring/early summer to take advantage of higher plant water use and consequently higher uptake of soluble Se.) The clay loam soil contained high levels of natural-occurring salts, including Se and B, and was classified as a Panoche soil (fine-loamy, mixed [calcareous], thermic Typic Torriorthents). General physical and chemical characteristics were as follows: 43% sand, 18% silt, 39% clay, bulk density of 1.23 g cm^{-3}, 0.6% organic matter, 0.58% total C, cation exchange capacity of 27.8 cmolc kg^{-1}, water content and water potential of 452 g kg^{-1} and –33 kPa, respectively. At 0–60 cm depth, pH was 7.9 ± 0.2, soil salinity (measured as extractable electrical conductivity [ECe]) averaged 7.0 ± 4.3 dS m^{-1}, soluble B 7.9 ± 5.3 mg L^{-1}, soluble Se 0.19 ± 0.15 mg L^{-1}, and total Se 2.4 ± 1.0 mg kg^{-1} DM (Table 1) Although redox potential [Eh] was not directly measured in this study, unpublished work on Se speciation in soil extracts by Bañuelos in these oxic alkaline soils shows that selenate is the dominant Se species in solution. The field site consisted of three blocks (A, B, C). Each block contained eight 110 m unelevated planting beds running west to east that were respectively separated by a distance of 30 cm. All beds were planted during early summer with the respective plant species in double rows with 15 cm distance between plants. In Block A (south of Block B), two beds were planted to green cabbage, two beds to Swiss chard, and four beds to savoy cabbage. In Block B (south of Block C), 2.5 beds were planted to broccoli, 1.5 beds to Swiss chard, two beds to red cabbage, and two beds to green cabbage. In Block C (north of Block B), only broccoli was planted on five beds, while the remaining three beds were left as unvegetated control beds. All plants were carefully sprinkler-irrigated throughout the growing season with good-quality water, consisting of the following chemical parameters (in mg L^{-1}): Cl (50), Na (37), Ca (16), Mg (10), S (9), K (3), B (< 1), P (< 1), Cu (< 1), Fe (< 1), Zn (< 1), Mn (< 1), Mo (< 1) and Se (< 1 μg L^{-1}) Co, Cr, Ni, Pb (not detected), EC of 0.4 (dS m^{-1}) and a pH of 7.6. Irrigation was based in part on weather data reported by California Irrigation Management Information System (CIMIS) located 5 km away at the UC Westside Research Field Station (see Supplemental Fig. 1). Soil samples were collected at 0–30 and 30–60 cm from a total of 32 locations within the three blocks (A = 12 sites, B = 12 sites, C = 8 sites). Sampling sites represented the eastern, western, and middle portions of the beds that ran east to west. Each soil sample was thoroughly mixed, oven-dried at 50 °C for 5 days, then ground in a Quaker City electric grinding mill, and sieved with a 1-mm screen. Water soluble fractions of Se and other elements were determined from a soil water extract with a ratio of 1:1 (w/v). Soil electrical conductivity (ECe) was determined using an Orion model conductivity meter, while chloride (Cl) was analyzed by potentiometric titration with silver nitrate using a Mettler Toledo titrator. Total Se was determined in a 1000 mg ground soil sample after wet acid digestion with HNO_3, H_2O_2, and HCl by inductively coupled plasma spectrometer (ICP-MS; Agilent 7500cx, Santa Clara, CA USA). Two internal soil standards (sediment collected from Kesterson Reservoir, CA with a total Se content of 7.5 ± 0.5 and 25.0 ± 0.9 mg kg^{-1}) were used as quality

Table 1
High and low salinity levels and concentrations of B, Cl, Na, S, and Se at two different soil depths for all vegetables grown in saline soils naturally laden with Se[†‡].

Vegetable	Soil Depth (cm)	Salinity Value Range	ECe ($dS\ m^{-1}$)	Total Se ($mg\ kg^{-1}$ DM)	Se	B	Cl ($mg\ L^{-1}$)	Na	S
Broccoli									
	0–30	Low	6.5 (1.3)	3.5 (1.0)	0.20 (0.08)	9 (3)	218 (79)	1204 (362)	1203 (221)
	0–30	High	15.1 (4.1)	4.4 (0.4)	0.30 (0.06)	19 (2)	1534 (619)	3625 (612)	2398 (683)
	30–60	Low	9.1 (1.8)	2.1 (0.6)	0.35 (0.09)	11 (3)	327 (175)	1977 (523)	1671 (287)
	30–60	High	17.6 (2.8)	2.1 (0.5)	0.41 (0.07)	20 (4)	1868 (626)	4332 (693)	2859 (236)
Red Cabbage									
	0–30	Low	2.6 (0.5)	2.3 (0.7)	0.15 (0.05)	3 (1)	85 (22)	401 (39)	410 (125)
	0–30	High	5.1 (0.9)	2.6 (1.1)	0.10 (0.03)	6 (3)	114 (45)	790 (234)	986 (175)
	30–60	Low	2.9 (2.1)	1.1 (0.2)	0.19 (0.06)	3 (1)	101 (13)	453 (230)	500 (155)
	30–60	High	7.1 (3.3)	1.8 (0.7)	0.17 (0.03)	8 (5)	279 (55)	1369 (236)	1369 (603)
Green Cabbage									
	0–30	Low	4.7 (0.4)	2.5 (0.5)	0.13 (0.07)	4 (0.3)	92 (8)	665 (101)	940 (85)
	0–30	High	5.8 (0.8)	2.4 (0.5)	0.10 (0.04)	7 (1)	144 (53)	977 (231)	1478 (368)
	30–60	Low	6.0 (0.7)	1.6 (0.1)	0.20 (0.05)	5 (0.2)	292 (45)	1042 (186)	1191 (135)
	30–60	High	7.5 (0.1)	1.6 (0.4)	0.31 (0.04)	7 (0.3)	281 (21)	1458 (131)	1889 (653)
Savoy Cabbage									
	0–30	Low	2.7 (0.7)	2.0 (0.2)	0.04 (0.01)	2 (0.2)	66 (9)	301 (55)	685 (450)
	0–30	High	6.2 (0.4)	3.0 (0.5)	0.10 (0.06)	8 (0.5)	193 (7)	1071 (105)	1958 (143)
	30–60	Low	3.1 (1.9)	1.1 (0.1)	0.09 (0.01)	2 (0.9)	42 (11)	446 (253)	862 (430)
	30–60	High	9.6 (1.1)	1.9 (0.1)	0.23 (0.11)	10 (2)	514 (103)	2086 (352)	2474 (518)
Swiss Chard									
	0–30	Low	2.8 (0.4)	2.2 (0.3)	0.06 (0.05)	2 (0.2)	73 (8)	278 (73)	671 (210)
	0–30	High	4.8 (0.4)	3.3 (0.3)	0.06 (0.01)	6 (0.6)	81 (3)	710 (120)	1395 (299)
	30–60	Low	2.4 (0.9)	1.1 (0.2)	0.14 (0.07)	2 (0.3)	50 (35)	325 (103)	592 (224)
	30–60	High	8.1 (2.1)	2.2 (0.2)	0.15 (0.11)	9 (2)	292 (46)	1633 (57)	2217 (371)

[†] Sampling occurred 25 days after transplanting, as described in methods and materials.

[‡] Values represent the means followed by the standard deviation in parentheses; ("n" values described in methods and materials). Statistical comparisons for ECe, soluble Se and total Se are presented in Supplementary Tables 1, 2 and 3, respectively.

control Se standards for soils. Selenium recovery rates were over 93% for each of them, respectively.

Normal agronomic practices were maintained for growing the crops, and no fertilizer was applied. After 86 days, the different plant species were harvested as follows: Five plants from each species were harvested from a 1-m^2 area surrounding each soil sampling site in each block, respectively, and bulked together as one sample. For each vegetable, there were the following numbers of composite samples ("n" value): savoy cabbage = 6, Swiss chard = 6, green cabbage = 4, red cabbage = 4, and broccoli = 12. The plant material was immediately cold stored in ice chests, and transported to USDA research facilities in Parlier, CA. Once at the USDA laboratory, the plant material was washed with deionized water, cut into small pieces, and plant material was divided into two equal parts for Se and total Se speciation analyses (described later). One part was stored at −80 °C until lyophilization took place with a Labconco Freezone 2.5 freeze dryer (Labconco Corp., Kansas City, MS), while the other part of the bulked sample was oven-dried at 50 °C for 5 days, ground to a fine powder in a UDY Cyclone mill equipped with a 1-mm mesh screen and further processed for total Se analyses, as described below.

Freeze-dried samples of plant material were ground by mortar and pestle, and chemical speciation of the soluble Se forms in aqueous proteolytic and non-proteolytic extracts were determined in plant samples that were freeze-dried and stored at −80 °C and analyzed by an Agilent 1200 HPLC equipped with a Hamilton PRP-X100 strong anion exchange column coupled to the Agilent 7500 CX ICP-MS (SAX-HPLC-ICP-MS), following our exact protocols originally described by Bañuelos et al. (2012). The selenoamino compounds were first identified by their retention times as compared to reference standards. A secondary confirmation was accomplished by spiking the proteolytic extracts with the individual reference standards. This step helped mitigate any matrix-induced changes to the retention times. Selenium extraction efficiency from freeze-dried samples was ~75%.

For plant total Se analysis, 500 mg of dried, ground shoot samples (edible portion of each plant species) were measured in triplicate using plant material from each bulked sample, digested with HNO_3, H_2O_2, and HCl, and analyzed by an inductively coupled plasma mass spectrometer (Agilent 7500 cx, Santa Clara, CA). The National Institute of Standards and Technology (NIST) Wheat Flour (SRM 1567) was used as the standardized quality control for plant samples. The Se recovery rates were over 94% for the wheat flour standard, which has a concentration of 1.1 ± 0.2 μg Se g^{-1} DM, while the method detection limit was 50 ng Se g^{-1} DM. Other elements (macro- and micronutrients) were analyzed from the plant digestate with the inductively coupled plasma spectrometer OES (Varian Vista-Pro, Santa Clara, CA).

Statistical analysis on plant and soil Se and B concentrations was performed using Sigma Plot 13 (SSI, USA), while one-way ANOVA with multiple pairwise comparison (Duncan's Method) was utilized to compare selenoamino acid contents, relationships between plant Se, S, and B and different soil parameters, and compare soil ECe, extractable and total Se at two different soil depths (0–30 and 0–60 cm) among the different vegetable species. Significance levels were expressed as $P < 0.05$ and $P < 0.001$.

3. Results

3.1. Soil chemistry

Soil sampling took place 25 days after transplanting, and variability in soil chemical properties was expected and observed under field conditions. Consequently, we divided our analytical soil data into two obvious salinity groups for this study: high and low (described below). For these tested field conditions, designations high and low are only used for this specific saline field site. Concentrations of soluble B, Cl, Na, S, and Se (presumably as selenate) are respectively presented for each depth (0–30 cm and 30–60 cm) for both high and low levels of salinity in Table 1. In these results, we are only reporting range values for salinity and Se at 0–30 and 30–60 cm for high and low salinity groups. Within the high salinity levels, salinity ranged from 4.8 to 15.1 dS m^{-1} at 0–30 cm and from 7.1 to 17.6 dS m^{-1} at 30–60 cm, while total Se ranged from 2.4 to 4.4 mg kg^{-1} DM at 0–30 cm and from 1.6 to 2.1 mg kg^{-1} DM at 30–60 cm, and soluble Se concentrations ranged from 0.06 to 0.30 mg L^{-1} at 0–30 cm and from 0.15 to 0.41 mg L^{-1} at 30–60 cm. Within the low salinity levels, salinity ranged from 2.6 to 4.7 dS m^{-1} at 0–30 cm and from 2.4 to 9.1 dS m^{-1} at 30–60 cm, while total Se ranged from 1.6 to 4.4 mg kg^{-1} DM at 0–30 cm and from 1.1 to 3.5 mg kg^{-1} DM at 30–60 cm, and soluble Se ranged from 0.06 to 0.20 mg L^{-1} at 0–30 cm and from 0.09 to 0.35 mg L^{-1} at 30–60 cm. There was a correlation coefficient of 0.43 (P value of 0.014) and 0.66 (P value of 0.0003) between salinity and soluble and total soil Se from 0 to 30 cm, respectively (n = 32, respectively). From 30 to 60 cm, there was a correlation coefficient of 0.56 (P value of 0.0008) and 0.55 (P value of 0.0013) between salinity and soluble and total soil Se, respectively (n = 32). Multiple pairwise comparison procedure (Duncan's Method) is shown for salinity (Supplementary Table 1), soluble soil Se (Supplementary Table 2) and total soil Se (Supplementary Table 3) at low and high soil salinity (ECe) sites at two different depths among the tested vegetables, respectively.

3.2. Tolerance observations and yields of vegetables

Visible heat or salt toxicity symptoms, e.g., necrosis on leaf margins, were not observed on any of the cool season vegetables grown in early summer. One likely consequence of the warmer temperatures was, however, observed on the broccoli plants; they did not produce florets under these warm summer growing conditions. All plant species appeared to be visually tolerant of the high ranges of salinity and B measured from 0 to 60 cm (Table 1). Fresh weight yields are reported in Table 2 for the different plant species on a metric ton (Mg ha^{-1}) basis. Among the vegetables, Swiss chard produced the lowest yields. As to be expected, fresh weight yields were lower than typical yields of the same vegetables grown as cool season crops under non-saline and low B conditions (data not shown), since vegetables for this study were grown in early summer (not their typical growing season) under excessive saline growing conditions. See Supplemental Fig. 2 to observe the physical appearance of each of the respective vegetables grown under field conditions.

3.3. Elemental mineral nutrient concentrations

The uptake of Se readily occurred in all vegetables as follows: broccoli 10.0 ± 3.7 mg Se kg^{-1} DM, green cabbage 13.1 ± 2.9 mg Se kg^{-1} DM, red cabbage 17.2 ± 6.4 mg Se kg^{-1} DM, savoy cabbage 11.0 ± 3.0 mg Se kg^{-1} DM and Swiss chard 4.8 ± 1.4 mg Se kg^{-1} DM. There were significant ($P < 0.05$) differences in total Se accumulation between Swiss chard and all of the other vegetable species (Supplementary Table 5). Bioaccumulation factors for total plant Se and mean soil soluble Se concentrations (plant available Se) from 0 to 60 cm soil depth were calculated as follows for each vegetable: broccoli (34), green cabbage (72), red cabbage (1 1 1), savoy cabbage (95), and Swiss chard (47). Our statistical analyses did not show consistent significant relationships between plant Se concentrations and concentrations of total or soluble Se, soluble S, or soil ECe, from 0 to 30 or 30–60 cm in soil (data not shown). The reason is likely due to the variability of soil ECe between the designated high and low soil salinity levels and the differences in soluble Se concentrations and soil salinity between 0 and 30- and 30–60-cm soil depths. Roots of the vegetables were exposed to variable levels of salinity and soluble Se between depths of 0–30 and 30–60 cm. The saline soils are natural sources for many elements, including macro- and micronutrients that are also available for plant uptake. Varied concentrations of naturally occurring mineral nutrients calcium (Ca), magnesium (Mg), potassium (K), S, sodium (Na), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), chlorine (Cl), B, and molybdenum (Mo) accumulated in the plant tissues from the respective vegetables grown in all blocks (Table 3). The ranges of macro- and micronutrient concentrations in these vegetables grown in these poor-quality soils varied as follows: Ca from a low of 0.9% in Swiss chard to a high of 3.4% in red cabbage; Mg ranged from low of 3,228 mg kg^{-1} DM in broccoli to a high of 7,390 mg kg^{-1} DM in Swiss chard; K ranged from a low of 2.1% in broccoli to a high of 4.4% in Swiss chard; S ranged from a low of 6,082 mg kg^{-1} DM in Swiss chard to a high of 20,319 mg kg^{-1} DM in green cabbage; Na ranged from a low of 3,041 mg kg^{-1} DM in savoy cabbage to a high of 34,133 mg kg^{-1} DM in Swiss chard; Zn ranged from a low of 26 mg kg^{-1} DM in broccoli to a high of 36 mg kg^{-1} DM in Swiss chard; Mn ranged from a low of 38 mg kg^{-1} DM in broccoli to a high of 258 mg kg^{-1} DM in Swiss chard; Fe ranged from a low of 120 mg kg^{-1} DM in broccoli to a high of 274 mg kg^{-1} DM in Swiss chard (Fe concentrations are often related to soil particle contamination); Cu ranged from a low of 11 mg kg^{-1} DM in broccoli to a high of 25 mg kg^{-1} DM in Swiss chard; Cl ranged from a low of 10,348 mg kg^{-1} DM to a high of 63,901 in Swiss chard; B ranged from a low of 152 mg kg^{-1} DM to a high of 239 mg kg^{-1} DM in green cabbage; and Mo ranged from a low of 1.3 mg kg^{-1} DM in Swiss chard to a high of 4.9 mg kg^{-1} DM in green cabbage. Absolute nutrient content was calculated for a fresh serving size of 36 g (one cup) for each tested vegetable and compared with the USDA Dietary Recommendations for Americans 2015–2020 (30–50 + years) (see Table 4). Analyses for heavy metals (e.g., Cd, As, Co, Hg, Pb) showed insignificant concentrations in any of the tested vegetables (data not shown). Not only were there insignificant concentrations in the soil, but the range of soil pH (7.8 to 8.2) reduces the probability of plant uptake if soluble heavy metals were present.

Table 2
Yields of tested vegetables grown under saline growing conditions.

Vegetable	Yield (Mg FW ha^{-1})	
Broccoli[†]	7.7[‡]	(1.7)
Green Cabbage	13.5	(2.6)
Red Cabbage	12.7	(2.2)
Savoy Cabbage	14.5	(1.7)
Swiss Chard	19.4	(2.1)

[†] Broccoli (whole plant) and no florets.

[‡] Values represent the means followed by the standard deviation in parentheses.

3.4. Selenium chemical speciation

Chemical speciation of the soluble Se in aqueous proteolytic and non-proteolytic extracts were determined in vegetable samples that were freeze-dried, ground, and stored at −80 °C. The selenoamino acids were extracted (as already described) and grouped as organic and inorganic Se. Total plant Se composition and percentages of selenoamino acids determined in the edible portion of each respective vegetable are presented in Table 5. Multiple pairwise comparison procedure (Duncan's Method) for Se speciation was shown in Supplementary Table 4 among the tested vegetable species. The chemical speciation of organic Se compounds in the vegetables were found to be diverse and occurring most frequently as organic Se forms C-Se or C-Se-C ranging between 46 and 65% organic Se, respectively. Secondarily, the inorganic form present was primarily the unreduced selenate (SeO_4) ranging from 35 to 54%. The two most bioactive monomethylated organic forms MeSeCys and gamma-glutamyl MeSeCys combined were as follows in each of the vegetables: 15% in savoy cabbage, 7% in broccoli, 15% in green cabbage, 21% in red cabbage, and 10% in Swiss chard, while $SeCys_2$ and inorganic selenite were detected under 9% and 5%, respectively. Plants from the *Brassiceae* family typically accumulate and synthesize Se into MeSeCys. It is not known if there is any relationship between MeSeCys concentrations produced within plants growing in

Table 3
Elemental concentrations in plant tissues of tested vegetables grown under saline growing condition[†].

Vegetable	B	Ca	Cd	Cl	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	S	Se	Zn
								(mg kg^{-1} DM)								
Broccoli	228	25,302	0.2	13,532	11	120	21,182	3228	38	4.6	13,687	0.66	2434	18,085	10.0	26
	(36)	(2951)	(0.0)	(2242)	(02)	(32)	(3036)	(351)	(11)	(0.7)	(2590)	(0.12)	(547)	(1985)	(3.7)	(4)
Green	239	31,514	0.3	17,721	15	215	23,526	4019	47	4.9	6746	1.04	2400	20,319	13.1	27
Cabbage	(37)	(5143)	(0.1)	(6521)	(06)	(98)	(6656)	(469)	(9)	(1.1)	(2288)	(0.17)	(173)	(2461)	(2.9)	(1)
Red	152	34,410	0.2	13,057	13	136	25,354	3641	64	3.4	11,397	1.04	2892	20,168	17.2	26
Cabbage	(56)	(3499)	(0.1)	(6647)	(03)	(37)	(6799)	(665)	(16)	(2.3)	(2382)	(0.16)	(378)	(2050)	(6.9)	(8)
Savoy	169	27,891	0.3	10,348	23	144	26,464	3437	44	3.7	3041	0.88	2934	20,542	11.0	26
Cabbage	(54)	(5575)	(0.1)	(5308)	(12)	(66)	(3882)	(829)	(11)	(1.3)	(1251)	(0.20)	(5 7 9)	(2824)	(3.0)	(6)
Swiss	186	9430	0.3	63,901	25	274	44,553	7390	258	1.3	34,133	1.02	2697	6082	4.8	36
Chard	(62)	(1570)	(0.1)	(6744)	(08)	(93)	(9346)	(1214)	(77)	(1.3)	(6547)	(0.26)	(470)	(2415)	(1.4)	(9)

[†] Values represent the means followed by the standard deviation in parentheses; ("n" values described in methods and materials).

these poor-quality soils. As a non-*Brassica* species, Swiss chard accumulated less Se and MeSeCys compared to the *Brassica* species. Future efforts are in progress by Bañuelos to evaluate the impact of varied levels of salinity and B on Se speciation changes within plant tissues.

The summation of the various forms of Se into their respective organic Se and inorganic Se compounds shows the following for each of the vegetables: 1) *broccoli* contained a total organic Se content of 56%, (53% organic Se [soluble] and 3.1% unknown*) and 44.5% inorganic Se; 2) *green cabbage* contained a total organic Se content of 59.2% (55% organic Se [soluble] and 3.7% unknown*) and 41.3% inorganic Se; 3) *red cabbage* contained a total organic Se content of 58.6% (55.8% organic Se [soluble] and 2.8% unknown*) and 41.4% inorganic Se; 4) *savoy cabbage* contained a total organic Se content of 64.9% (58.8% organic Se [soluble] and 6.1% unknown*) and 35.1% inorganic Se; and 5) *Swiss chard* contained a total organic Se content of 46.2% (41.3% organic Se and 4.9% unknown*) and 54% inorganic Se. (*Note: This unknown peak occurred late in the chromatographic elution and was likely protein-bound Se composed of SeCys and SeMet and not completely broken down by the proteolytic enzyme).

4. Discussion

Our results demonstrate that we can naturally produce Se and other mineral-nutrient-biofortified vegetables from saline and B California soils naturally laden with inorganic Se and high concentrations of soluble S. Other natural biofortification studies in progress in China, i.e., Hubei Province (Yin et al., unpublished), have similarly demonstrated natural Se biofortification in food crops, but grown in non-saline soils with high levels of naturally occurring Se. In central California, with over 200,000 ha containing soils with naturally occurring Se, albeit found in conjunction with potentially toxic levels of salt, B, and other elements, only crops that possess salt and B tolerance can be considered for natural Se biofortification strategies in this area. In our present study, the accumulated Se in the different crops may have inadvertently contributed to their successful growth under poor growing conditions since others have reported that elevated plant Se levels enhance salt tolerance (Bocchini et al., 2018). Despite the high levels of soluble S in the soil, a competitive ion for Se uptake, Se absorption by the cruciferous vegetable species was still substantial. We attribute the high uptake of Se to the relatively high concentrations of soluble Se in the soil (measured 25 d after transplanting) that were as high as 0.41 mg Se L^{-1}, despite high soluble S as high as 2,859 mg L^{-1}. In addition, the high levels of soil salinity have the strong ability to hold on to soluble forms of Se and other soluble elements (e.g., potentially toxic B), which reduces a predictable uptake of soluble Se as selenate and may also account for the correlation observed between soil ECe and soluble Se in the soil. In these typical west side soils of central California, salinity is generally associated with naturally occurring Se. Under non-saline growing conditions, we would expect to observe a significant positive relationship between soluble Se in the soil and plant Se concentrations. The lack of significant statistical relationships between plant Se content and the tested soil parameters indicate the difficulty in predicting plant Se levels for Se biofortification under both variable soil Se conditions and high B and saline conditions. The predictable availability of soluble Se will constantly be in a state of fluctuation due to plant uptake of Se and lateral and vertical movement of soluble Se in the soil under irrigated field conditions. With our tested vegetables, the cabbages had the greatest Se bioaccumulation factors: red cabbage > savoy cabbage > green cabbage > Swiss chard > broccoli, and consequently may be the more ideal vegetable to grow for natural biofortification

Table 4
Recommended absolute amounts of nutrients by USDA Dietary Guidelines 2015–2020 for 31 to 50 year plus Americans compared to recovered absolute amounts of nutrients measured in the respective tested vegetables grown in this study[†].

	Recommended amounts for:				Absolute amounts in the tested vegetables:				
Nutrient	Female 31–50	Male 31–50	Female 51 +	Male 51 +	Broccoli	Green Cabbage	Red Cabbage	Savoy Cabbage	Swiss Chard
Calcium (mg)	1000	1000	1200	1200	91[17] [‡]	113[14]	124[16]	100[13]	34[18]
Iron (mg)	18	8	8	8	0.4[0.3]	0.8[0.2]	0.5[0.3]	0.5[0.2]	1.0[0.6]
Magnesium (mg)	320	420	320	420	12[9]	14[4]	13[6]	12[10]	27[29]
Phosphorus (mg)	700	700	700	700	9[24]	9[9]	10[11]	11[15]	10[17]
Potassium (mg)	4700	4700	4700	4700	76[117]	85[61]	91[88]	95[83]	160[136]
Sodium (mg)	2300	2300	2300	2300	49[10]	24[6]	41(10)	11[10]	123[77]
Zinc (mg)	8	11	8	11	0.1[0.1]	0.1[0.1]	0.1[0.1]	0.1[0.1]	0.1[0.1]
Copper (mcg)	900	900	900	900	40[16]	54[7]	47[6]	83[22]	90[64]
Manganese (mg)	1.8	2.3	1.8	2.3	0.14[0.08]	0.17[0.06]	0.23[0.09]	0.16[0.07]	0.93[0.13]
Selenium (mcg)	55	55	55	55	36[1.1]	47[0.1]	62[0.2]	40[0.3]	17[0.3]

[†] Based upon a serving size of 36 g fresh weight of the respective vegetable.

[‡] Values within brackets indicate the typical absolute amounts of the respective nutrients in crops grown in non-saline soils, as reported in the USDA Agricultural Research Services Food Data Central Database. Reported values are based on a serving size of 36 g fresh weight of the respective vegetable.

Table 5
Total Se and selenoamino acid percentages measured in the different tested vegetables grown in saline soil and naturally high levels of Se.

Vegetable	Total Se (mg kg^{-1} DM)	$SeCys_2$	MeSeCys	Selenite	SeMet	γ-glutamyl MeSeCys	Selenate	Unknown
					(%)			
Broccoli	10.0[†]	6.9	6.3	2.4	38.9	0.6	42.1	3.1
	(3.7)	(2.7)	(2.6)	(1.2)	(3.4)	(0.3)	(9.9)	(2.1)
Green Cabbage	13.1	7.7	13.7	2.9	32.8	1.3	38.4	3.7
	(2.9)	(1.2)	(2.3)	(1.2)	(6.0)	(0.9)	(7.8)	(1.6)
Red Cabbage	17.2	5.3	18.9	3.8	29.7	1.9	37.6	2.8
	(6.9)	(1.8)	(4.0)	(1.2)	(6.5)	(1.5)	(11.4)	(1.4)
Savoy Cabbage	11.0	7.3	11.4	4.7	36.5	3.5	30.4	6.1
	(3.0)	(1.3)	(1.6)	(1.8)	(7.4)	(1.5)	(9.1)	(2.1)
Swiss Chard	4.8	8.9	6.7	3.7	24.1	1.6	50.8	4.9
	(1.4)	(2.4)	(1.9)	(1.2)	(7.4)	(0.9)	(8.9)	(1.2)

[†] Values represent the means followed by the standard deviation in parentheses; ("n" values described in methods and materials). Statistical comparisons for Se speciation are presented in Supplementary Table 4.

under these adverse growing conditions. Movement of soluble Se will also be strongly promoted by irrigation practices and the form of irrigation used. Practicing sound water management with surface or subsurface drip can minimize the downward or lateral movement of soluble Se in the soil. In this study, excessive sprinkler irrigation was avoided because water application rates were always determined based upon evapotranspiration losses and other weather data reported by CIMIS (see Supplemental Fig. 1). While irrigation application can affect Se retention in soil (Se partitioned on clay particles), Se can desorb following temporary changes in pH or redox state, or even irregular precipitation activities. These fluctuating influences are important to note for natural Se biofortification strategies because the ever-changing status of soluble Se in the soil makes it difficult to predict plant tissue levels of Se unless consistent and numerous determinations of soil soluble Se takes place throughout the growing season. The wide range of concentrations measured for Se and the other nutrients in the different vegetables is clearly a result of growing the different vegetables in saline fields with variable levels of Se and other elements. With this natural Se biofortification strategy practiced in this saline-, B-, and element-rich soil, we also clearly observed a wide range in concentrations of macro- and micronutrient biofortification in the vegetables. Under variable field conditions, especially in regards to salinity, one can expect variable soluble concentrations of other nutrients, e.g., Na, K, and Cl, in the crops. Despite the variance, these results indicate that the intake of other essential nutrients, i.e., Ca, Mg, Na, S, B, and Cl, can also be slightly increased (< 10%) with the consumption of these Se-biofortified vegetables produced under these tested field conditions. Table 4 shows typical absolute values of the measured nutrients in the same vegetable species grown under non-saline conditions. In regard to health safety associated with intake of other accumulated nutrients via consumption of one fresh serving of the respective tested vegetables, Table 4 shows the calculated absolute amounts of Ca, Fe, Mg, P, K, Na, Zn, Cu, and Mn compared to the daily absolute amounts of nutrients recommended by USDA Dietary Guidelines for 2015–2020. Among all the nutrients tested, only Se intake could be significantly enhanced by consumption of Se-biofortified vegetables.

Consumption of a typical fresh portion of any of the vegetables would be safe, as seen below, despite the variable Se concentrations (ranging from 4.8 to 17.2 mg kg^{-1} DM) in the different vegetables evaluated in this study. For example, if we assume that one cup (typical serving size of 36 g fresh weight) was respectively consumed of the biofortified vegetables, the following amounts of absolute Se (in µg) would be ingested for each fresh vegetable based upon the mean total Se concentrations reported on a dry weight basis in Table 5 (and assuming a 90% water content in fresh tissue): broccoli (36 µg), green cabbage (47 µg), red cabbage (62 µg), savoy cabbage (40 µg), and Swiss chard (17 µg). At these levels, and considering the reported standard deviation associated with total plant Se, safe amounts of Se would range from 12 to 87 µg Se $serving^{-1}$ by fresh consumption of the tested vegetables. Bioavailability of Se for intestinal absorption will strongly depend on the bioaccessible Se available after ingestion of Se-enriched plant material. Hence, the speciation of Se in plant tissue is important to know for understanding the efficiency of Se absorption. For all the tested cruciferous vegetables, we found that the speciation of Se was generally similar as follows: SeO_4 > SeMet > MeSeCys > $SeCys_2$ > SeO_3 > gamma-glutamyl MeSeCys. Since these vegetables are all *Brassica* species (except Swiss chard is a related *Cruciferae*), they all have a strong affinity for S. Consequently, it is expected that all of them would generally exhibit a similar Se speciation when grown in a soil environment containing naturally occurring Se. The identification of organic Se compounds are potentially of great interest for health-related issues, since MeSeCys and gamma-glutamyl MeSeCys, as monomethylated Se compounds, were reported earlier to be the selenoamino acids most significant for human health and potentially involved with chemoprevention activities (Finley et al., 2001; Ip & Ganther, 1992). In most of the vegetables (except Swiss chard), the total organic Se content was always greater than the total inorganic Se content. This result indicates that bioaccessibility of Se for human absorption will likely be greater with organic forms of Se than inorganic forms of Se. In this regard, Du Laing et al. in Belgium are investigating the bioaccessibility of Se from different forms of Se in different Se-enriched food products (Sun, Van der Wiele, Alva, Tack, & Du Laing, 2017). In this study, SeMet was the dominating organic Se species identified in all our tested vegetable species, as to be expected with most non-Se-accumulating plant species. Future studies need to evaluate the effects of processing and cooking on Se speciation changes within Se-enriched plant products, i.e., SeMet in Se-enriched plant products (Lu et al., 2018). Studies by Bañuelos et al. (unpublished) are currently in progress on evaluating the influence of cooking on Se speciation changes in soups made from these tested Se-biofortified vegetables.

We have successfully produced natural Se-biofortified vegetables from typical cool-season crops grown on unproductive and high saline- and B-laden field sites during late spring/summer growing conditions. The apparent salt and B tolerance and accumulation of Se exhibited by these tested cruciferous vegetable species clearly shows that natural Se biofortification can occur even under adverse growing conditions if soluble Se is present in the soil. Longer-term studies are needed to examine Se uptake and potential differences in speciation changes in crops grown under a natural biofortification strategy on a sustained basis. Temporarily bound Se within the soil profile will be gradually released from particles or organic carbon and be available for uptake by plants. Under field conditions, there is a large reservoir of naturally occurring Se in the soil, and it will likely never be depleted from growing crops as part of any biofortification strategy. However, the amount of soluble Se available for plant uptake will constantly be in a state of fluctuation as affected by irrigation practices, soil chemical activities, and plant uptake of Se, especially with repeated plantings on the same field site. Consequently, it will be difficult to accurately predict plant Se concentrations over time and especially under high saline, B, and soluble S growing conditions. This unpredictability illustrates the importance of accurately monitoring Se content and Se speciation in crops grown under a natural biofortification strategy, as well as compiling harvested material into a single large batch from variable field sites for final processing (drying and grinding). Accurate speciation analysis on each crop must be performed on freshly-consumed biofortified vegetables, and it is recommended that the same analyses be performed on any of the vegetables further processed and prepared as a dried, ground, and powdered food product.

5. Conclusion

Natural Se and nutrient biofortification of cruciferous vegetables can occur when they are grown in poor-quality soils high in salinity, Se, and B. The plants will absorb naturally occurring Se, as well as other soluble elements present in the soil. Importantly, heavy metals were not significantly present in these high-pH soils of central California, and consequently insignificant amounts were detected in any of the vegetables. In general, natural Se biofortification can be an environmentally friendly strategy for producing Se-enriched nutraceutical food products, especially considering environmental concerns associated with the excessive accumulation or runoff of Se with excessive applications of soil-applied inorganic Se (a strategy practiced outside the USA). After careful analysis for total Se and for the different selenoamino acids, consumption of Se-enriched vegetables or vegetable food products can be a nutritionally sound strategy for increasing Se intake in Se-deficient areas where human health and Se-deficient disorders can more frequently occur. Bioaccessibility for effective human absorption of Se needs to be examined as it relates to selenoamino acid species and content within the biofortified food product. Future studies should also examine the impact of food product processing and preparation on changes of Se speciation or losses of Se as SeMet in biofortified food crops and products. Those growing crops on naturally rich Se soils must consider that soil Se variability, changes in soluble soil Se concentrations, and Se speciation changes occurring over time in the soil make it more difficult to predict or produce naturally Se-biofortified products with consistent Se concentrations on a sustained basis under field conditions. Periods of laying fields fallow in between cropping seasons to dry out soil in these arid environments will help wick soluble minerals, including selenate, back to the upper soil horizon. Importantly, under these adverse growing conditions, the planting of salt- and B-tolerant vegetable species and S-loving crops, i.e., *Brassica* crops, with specific SeCys selenoamino acid methylation enzymes (methylselenocysteine methyl transferase), may also determine the rate at which Se accumulates via the S pathway and produces a variety of selenoamino acids.

Credit authorship contribution statement

Gary S. Bañuelos: Conceptualization. **John Freeman:** Formal analysis. **Irvin Arroyo:** Formal analysis, Methodology.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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