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Title: Biofortification of baby leafy vegetables using nutrient solution containing selenium

Running title: Baby leafy enriched with Selenium

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Abstract

BACKGROUND: Biofortification of vegetables is an important innovation technique in the horticultural sector. Vegetables can be a vector of different minor elements that have beneficial effects on human health. Selenium (Se) is an important element for human nutrition and plays a significant role in defense mechanisms. The aim of this work was to investigate the effect of Se in the nutrient solutions on the crop biofortification ability, yield, and quality parameters of four baby leaf vegetables destined to the minimally processed industry. Experiments were performed on lamb's lettuce, lettuce, rocket, and spinach. These crops were cultivated in the floating systems with nutrient solution enriched with 0, 2.6, 3.9 and 5.2 µmol L^{-1} Se provided as sodium selenate. **RESULTS.** At harvest, selenium concentrations, yield, nitrate concentration, sugars, and some mineral elements were measured. Data collected and analyses showed that yield, nitrate, sucrose, and reducing sugars were not affected by Se treatments, even if varied among species. Se concentrations linearly increased in leaves of different species by increasing the Se concentration in the nutrient solution. Rocket was the species with the highest accumulation ability and reached the concentration of 11 μg g^{-1} FW Se in plants grown with 5.2 μmol L^{-1} Se.

CONCLUSION. Floating system with Se enriched nutrient solution is an optimal controlled growing biofortification system for leafy vegetables. The accumulation ability decreased in different species in the following order rocket, spinach, lettuce, lamb's lettuce, highlighting a crop dependent behaviour and their attitude to biofortification.

Key words: fresh-cut, minimally processed, ready to eat, salads, sugars, yield, floating system, functional foods

Introduction

Leafy vegetables are primary source of antioxidants, vitamins, minerals, fiber, and other nutritional compounds in the human diet[1]. Leafy vegetables can be harvested and commercialized as adult salads or as baby leaf. The main differences between them are the harvesting stage and the nutritional composition. Baby leaf vegetables are harvested at ~~in~~ the very young stage when they have 3-5 fully expanded leaves and 10-15 cm of height. These vegetables are mainly commercialized as ready to eat or fresh cut vegetables or minimally processed. Baby leaf vegetables are usually grown in greenhouse or tunnels in soil or hydroponic systems such as floating system[2]. Leafy vegetables should be a constant component in a healthy diet[3] and the composition can be influenced by the nutrient availability. Some elements can be increased in the soil or in the nutrient solution for increasing their concentration in the edible leaves of vegetables[4]. This procedure is also called biofortification and represents the enrichment of nutrients in food for improving the human nutrition.

Selenium (Se) is a mineral involved in several biological functions in plants and animals. Se is also cofactor of important enzymes such as thioredoxin reductase and glutathione peroxidase, which have a protection role against oxidative stresses. Se is a component of these enzymes as selenocysteine in plants[5], but there are many others forms of organic Se such as selenomethionine, selenomethylselenocysteine, selenocystathionine, selenomethylselenomethionine, dimethylselenopropionate, dimethylselenide, and dimethyldiselenide[6] . The main function of Se is as antioxidant with tissue protection function in plants, human, and animals[7,8]. The antioxidant ability of Se is particularly important in the protection of the cell membrane and prevent cancer formation in the human[9]. However, the anticarcinogenic activity and its beneficial effect in human health can be obtained in a very narrow range of Se concentrations. Higher concentrations respect the advisable daily intake can be dangerous for the consumer's health[10,11,12]. The Se concentration in the edible plant parts depends on its concentration in soil and crop uptake ability. The Se available in soil depends on its forms, microflora, biological, chemical, and physical characteristics. In agricultural systems with low Se concentrations, it can be directly added to fertilizers[13,14]. However, this strategy must be carefully carried out, because high Se supply can induce accumulation in soil with the risk of excessive crop uptake and subsequent high Se concentration in the edible products[15].

The Se crop biofortification can be also achieved by foliar fertilization, avoiding the soil accumulation[16,17]. High Se levels in soil are not only dangerous for human health but they can also have negative effect on crop productivity with or without showing visual toxicity symptoms[18].

Controlled cultivation such as soilless systems and in particular floating system can represent easy methods for modulating the Se availability and thus the crop uptake. Positive results have been reported for tomato grown with nutrient solution enriched with Se (0-4 µmol L^{-1} SeO_2) and administrated through fertigation in soilless growing system[19].

Floating system is the simplest hydroponic cultivation system composed by a tank filled with a nutrient solution and floating panels. Vegetables are sown and grown on the nutrient solution. The addition of Se directly in the nutrient solution in floating cultivation system could allow producing leafy vegetables enriched avoiding excessive concentration in leaves[20,21]. The stagnant nutrient solution is isolated in the cultivation tank, and it can allow an higher control level of the Se uptake by modulation the Se concentration in the nutrient solution.

The aim of this work was to study the effect of Se concentration in the nutrient solutions on the crop biofortification ability, yield, and quality parameters of four baby leaf vegetables destined to the fresh-cut industry. The hypothesis of this work was that the application of Se by nutrient solution could increase the Se leaf content in leafy vegetables, which can be carriers of Se in human diet. The accumulation ability of vegetables, at different Se concentrations in the nutrient solution, and the amount of leafy vegetables necessary for providing the recommended daily allowance (RDA) were investigated.

MATERIALS AND METHODS

Plant materials, growing conditions, and Se treatments

Lamb's lettuce (*Valerianella olitoria* L. [*Valerianella locusta* (L.) Laterr.] cv. Trophy), lettuce (*Lactuca sativa* L. cv. Chiara), rocket (*Diplotaxis tenuifolia* L. var. Frastagliata), and spinach (*Spinacia oleracea* L. cv. Pungi F1) seedlings were grown under greenhouse covered with plastic film Centre of Advanced Technologies in Greenhouse (CETAS, Tavazzano – Lodi) of the University of Milano. Seeds were sown in polystyrene trays (32.5 cm x 51.5 cm) with 228 holes with perlite (density was about 4000-5360 seeds m^{-2} considering about 3-4 seeds per hole). After germination, the trays were transferred in tanks containing 700 L with 4 trays for each tank.

The Se was directly added in NS from beginning of the cultivation by dissolving the sodium selenate salt (Na_2SeO_4).Se concentrations in the NS were 0, 2.6, 3.9 or 5.2 µmol L^{-1}.

The nutrient solution contained 6.5 N-NO_3, 0.75 P, 4 K, 1.75 Ca, 0.85 Mg, 4.7 Na, 4.0 Cl, 1.3 S (concentrations are expressed in mmol L^{-1}), while micronutrients were provided at Hoagland's concentration (expressed in µmol L^{-1}): 20 B, 40 Fe, 1.5 Cu, 5 Zn, 10 Mn.

pH, EC and temperature of the nutrient solutions

During cultivation, pH and EC of the NS were constantly monitored. The EC of NS was 1.6 dS m^{-1} at beginning of experiment and pH was adjusted to 6.0 with 0.11 mL L^{-1} (77 mL/700 L) sulfuric acid (9.6 g Kg^{-1} Sigma-Aldrich, Italy), during cultivation water was not added. The pH showed variations and declined in all treatments until 4.65 at the end of growing cycle (Fig. 1A). During the whole cultivation the EC was stable with an increase at the end of cycle up to reach 1.75 dS m^{-1} (Fig. 1B). The temperature of the NS measured at 11 a.m. ranged from 25 to 13 °C, since the cultivations were in September-October (Fig. 1C).

Four cultivation tanks for four species were placed in four randomized blocks in greenhouse. The oxygenation of nutrient supply was performed by bubbling air using an air compressor. The leaves were collected at commercial stage for baby leaf production. The temperature and the solar radiation outside greenhouse were monitoring during the whole experimental period (five weeks), with temperature ranging from 12 to 27°C and radiation from 3945 to 9707 KJ m^{-2} d^{-1}.

Yield, nitrate content, sucrose and reducing sugars determination

At harvest, yield was determined and expressed as kg m^{-2} of edible biomass. Leaf nitrate concentration was measured by spectrophotometry using the salicylic-sulphuric acid method. For each sample 100 mg of DW was ground and placed in icon glass with 10 mL of distilled water. Dry leaf powder was shaken for 2 h at room temperature and then 10 mL were centrifuged for 15 min at 4000 rpm. The supernatant was taken, and 0.2 mL were added to 0.8 mL of 0.5 g kg^{-1} salicylic acid in sulphuric acid. Samples were placed on the stirring machine and 30 mL of 1.5 $mol \cdot L^{-1}$ NaOH were added. Cooled samples were read at 410 nm[22] (Cataldo et al., 1975). The nitrate concentration was determined using calibration standards containing 0, 1, 2.5, 5, 7.5 and 10 $mmol \cdot L^{-1}$ KNO_3.

Sucrose and reducing sugars were determined, about 2 g of samples were homogenized in a mortar using water as buffer. The insoluble materials were separated by centrifugation for 5 min at 10000 rpm.

The sucrose determination was carried out by adding 0.2 mL of aqueous extract obtained from 2 g of samples with 0.2 mL NaOH 2 N and incubated in a water bath for 10 min at 100 °C, then 1.5 mL of hot resorcinol buffer was added and samples were incubated in a water bath at 80 °C for other 10 min. Resorcinol solution was prepared by mixing 250 mL HCl 3 g kg^{-1}, 90 mg thiourea (Sigma, Italy), 35 mg resorcinol (Sigma, Italy), 25 mL acetic acid, and 10 mL distilled water. Sample readings were spectrophotometrically performed at 500 nm, using sucrose standard solutions containing 0, 0.5, 1, 1.5, and 2 mmol L^{-1}.

The reducing sugars were performed using 0.2 mL of aqueous extract obtained from 2 g of samples that were added to 0.2 mL of dinitrosalicylic acid (DNS). Reactions were heated at 100 °C for 5 min, then 1.5 mL of distilled water was added, and absorbance readings were taken at 530 nm. Reducing sugars were measured and reported as glucose equivalent (standards: 0, 1, 2, 3, and 4 mmol L^{-1} glucose).

Se and mineral determinations

Fresh harvested leafy vegetables were placed in ventilated oven at 40 °C until a constant weight. About 400 mg dry weight (DW) was mineralized in 5 mL 14.4 M HNO_3, clarified with 1.5 mL 3.3 g kg^{-1} H_2O_2. The mineralized material was solubilized in 5 mL 1 M HNO_3 and filtered on a 0.45-µm nylon membrane. Mineral concentrations (K, Ca, Na, Mg, B, Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, and Pb) were measured by inductively-coupled plasma techniques (ICP-MS; Varian 820-MS, ICP Mass Spectrometer).

Statistical analyses

The data reported in tables and figures are means with standard errors (*n*=4). The significance of the effect of nutrient concentration was determined by two-way ANOVA. Differences among treatments were determined by Tukey's multiple comparison test (P=0.05).

The principal component analysis (PCA) was carried out to the identification of the mineral element distributions in baby leaf in relation to species. PCA with eigenvalues > 2, explaining more than a single parameter alone, were extracted. For these principal components, the Varimax rotation was applied on the obtained factor.

RESULTS

Selenium content in baby leaf and recommended daily allowance (RDA)

Leafy vegetables are mostly consumed as raw fresh vegetables, Se concentrations were expressed on both dry and fresh weight basis (Fig. 2A, B). Se content in the fresh biomass generally increased with the addition of Se in the nutrient solution. In lettuce, lamb's lettuce, and rocket the Se concentration in leaves increased. The linear regression analysis between Se concentrations in the NS and Se concentrations was significant for p= 0.0024 in lettuce, p= 0.0003 in rocket, p= 0.0146 in spinach, and p= 0.0002 in lamb's lettuce. Spinach plants grown at 2.6 and 3.9 µmol L^{-1} Se showed similar concentrations (2.5 µg g^{-1} FW Se). The highest Se concentration was found in rocket grown at 5.2 µmol L^{-1} Se (12 µg g^{-1} FW Se) followed by spinach, lettuce, and lamb's lettuce. The Se concentration in baby leaf vegetables expressed on dry weight basis followed a similar trend observed for fresh weight. Se concentrations were higher in the baby leaf cultivated in nutrient solution containing 5.2 µmol L^{-1} Se in all species compared to control. Rocket showed the highest Se concentration at 5.2 µmol L^{-1} with a value of 56 µg g^{-1} DW Se (Fig. 2B). Lettuce and spinach at highest Se concentration showed an average of 15 µg g^{-1} DW Se, while lamb's lettuce slightly lower of 10.5 µg g^{-1} DW Se (Fig. 2B).

The average amount of studied baby leaf vegetables required for the satisfaction of Se RDA in non-biofortified treatments was about 358 g (Fig. 2C). The enrichment of Se strongly reduces the required amount. Rocket was able to uptake higher amount of Se at the different concentrations if compared with other species. Therefore, rocket can satisfy the RDA just with 9 g per day if harvested from 5.2 µmol L^{-1} Se treatment. At this concentration, the amount of the other baby leaf required for the satisfaction of the RDA was in average 19.9 g on fresh basis.

Yield, nitrate, sucrose, and reducing sugars

Two-way ANOVA revealed that there was no significant interaction on yield among Se treatments and species (Tab. 1). Differences were not found among Se concentrations (Fig. 3A).

Nitrate data subjected to two-way ANOVA showed that the interaction among Se treatments and species was not significant and differences were not significant found among Se concentrations. On the contrary, significant differences were observed among species. Spinach showed the lowest nitrate concentration with a value of 818 mg kg^{-1} FW, while lettuce and lamb's lettuce had intermediate concentrations with 2099 and 2145 mg kg^{-1} in average, respectively. Rocket, instead, showed the higher concentrations with 3954 mg kg^{-1} as average of all Se treatments (Fig. 3B).

As reported for nitrate, sucrose and reducing sugars were not statistically affected by Se concentrations. Significant differences were found among species as well as the interaction Se × Species was statistically significant (Tab. 1).

Se treatments in lettuce and spinach did not influence the concentration of reducing sugars, data ranged from 2.42 to 3.23 mg g^{-1} FW. In rocket, reducing sugars were about 1.9-fold higher in 3.9 µmol L^{-1} and 1.5-fold higher in 5.2 µmol L^{-1} than control. Lamb's lettuce Se showed a reduction of 41% in 3.9 µmol L^{-1} and 37% in 5.2 µmol L^{-1} compared to control (Fig. 3 C).

Sucrose concentration was affected by Se treatments only in spinach, which was higher in 5.2 µmol L^{-1} Se treatment than control by about 550% (Fig. 3D). No differences were observed at the increase of Se in the other species.

PCA and correlation analysis between measured variables

The PCA analysis indicated that mineral elements analysed had a distinct response pattern in rocket, lamb's lettuce, lettuce, and spinach. Two significant components were produced that, together, explained 69.39% of the total variance of data. Component 1 explained 43.5% of the total variance, including Se, Ca, Na, Mg, Mn, and Zn; K, B, Fe, and Cu were associated to the second component (25.8% of the variance, Fig. 4).

When the association among the parameters analysed and two main components were identified, the scores assigned to individual species with respect to each mineral element were analysed to identify common trends. Moreover, a scatter plot of scores of different treated samples in a Fact.1/Fact.2 score space is reported in the Fig. 4. Scores are marked by sampling species to identify possible differences among vegetables studied. In the PCA plot a different distribution of the data with respect to species can be clearly observed. A net difference was detected among rocket and other species in relation to Se treatments (Fig. 4).

The correlation matrix revealed that significant correlations were observed by pooling together the data of all species. The Se was positively correlated with Ca, Na, B, nitrate, and negatively correlated with K and Mn (Tab. 2). It interesting that Se directly or indirectly affected the mineral elements uptake.

DISCUSSION

This work was planned to produce Se enriched baby leaf vegetables for providing functional foods in the human diet. The biofortification of leafy vegetables with Se must be accurately performed for avoiding high concentrations that could be phytotoxic for plants and dangerous for human health. In open field, the crop enrichment can be mainly obtained through the fertilization using fertilizers containing Se applied to the soil or as foliar spraying[14,23]. In closed-loop hydroponic, such as floating system, the Se can be included as component of the nutrient solution and constantly monitored during cultivation. The use of nutrient solutions enriched with Se has been tested in a wide range of vegetables, showing promising practical applications[20,24,25]. The Se did not influence the chemical parameters of the nutrient solution such as pH or EC.

The Se concentrations found in the studied biofortified species were similar to those observed in analogous research works reported in literature. In cultivated rocket (*Eruca sativa* Mill.) the application every two days with solution containing Se ranging from 5 to 3000 µmol L^{-1} confirmed that this species has a high accumulation ability. In treatment with 100 µmol L^{-1} the Se in the shoot reached the 6.5 mg g^{-1}. Cultivated and wild rocket (*Diplotaxis tenuifolia*) have different accumulation ability and the latter at the same Se concentration showed higher accumulation in the range of 10-20 µmol L^{-1} [26]. In lamb's lettuce Se biofortification was studied for enhancing crop tolerance against high temperature stress. The application of 264 µmol L^{-1} (50 mg Se L^{-1} applied as Na_2SeO_4) improved the crop stress tolerance and the Se concentration in shoot varied from 19-56 mg g^{-1} DW depending on the application method, foliar or soil[27]. A biofortification study carried out on different accessions of lettuce demonstrated that Na_2SeO_4 is the preferred uptake form and Se accumulation ranged from 3.8 to 7.5 µg g^{-1} DW in treatment with 15 µmol L^{-1} [28]. Se concentrations found in leafy vegetables grown in the highest Se concentration were similar to those found in other vegetables species such as lettuce or chicory (*Chicorium intybus* L.) cultivated with 0.5 mg Se L^{-1} [20]. Similar Se accumulations were also observed in edible parts of cabbage, garlic, onion, radish, or spinach[29,30]. Recently, biofortification has been succesully carried out on wild species such as *Rumex acetosa* L., *Plantago coronopus* L., and *Portulaca oleracea* L.[31]

The recommended dietary allowance (RDA) for Se has been defined to be 55 μg day^{-1} for adults 32. In human nutrition, Se deficiency has been associated to diets with less than 0.1 mg Se kg^{-1} in foods. On the contrary diets with Se concentration higher than 1 g Se kg^{-1} can cause toxicity and selenosis in humans[33]. The higher limit of Se intake for adult has been set at 400 μg day^{-1} (WHO and FAO of the United Nations, 2004) There are several geographical areas were the Se in soil is limited and can lead to produce with lack of this element in agricultural foods. In the human diet the Se is better absorbed if incorporated in organic molecules compared to mineral Se supplement[34]. The floating system is an optimum hydroponic method for increasing leafy vegetables because the Se concentration can be constantly monitored in the nutrient solution and in edible parts, avoiding excessive accumulation in plants. Biofortified baby leaf can easily provide the RDA value with progressively smaller amounts of vegetables with the increase of Se in the nutrient solution. The amount of baby leaf that can be used for satisfying the RDA depends on Se concentrations and the accumulation ability of the species used. Minimally processed industries can use biofortified leafy vegetables for preparing bags with single leafy vegetable species or mixed ones that can satisfy the RDA of the consumer.

Se treatments affected the ionome of baby leaf species, overall comparison and correlation analyses revealed that some mineral elements were positively or negatively correlated with Se. In particular, Se showed positive and significant pooled the data of four species for Ca, Na, B, and nitrate. This positive correlation was also found in asparagus treated with sodium selenite[35]. A positive correlation was also found between Se and Na. Analogous results, were found in black gram (*Vigna mungo* L.) plants[36]. However, the interaction of Se on the uptake with other elements depends on the Se concentration applied. In cultivated rocket the concentration of P, K, Mg and Ca increased shoot grown with 5 μmol L^{-1} selenate, while at 100 μmol L^{-1} selenate a significant reduction in P, K, and Mg concentrations were recorded. In tomato fruit analogous were observed at higher Se concentration[37].

The effect of Se was studied on crop yield as well as on leafy vegetable quality parameters. Results demonstrated that Se treatments did not affect yield in all four species used. However, different species and diverse environmental conditions can lead to different results. An increase of yield in Se treatments was observed in chicory and lettuce[20], while no yield change was observed in garlic and onion[38] or asparagus[35] foliar sprayed with Se.

In our study, beside the Se accumulation also the effects of Se treatments on quality parameters were considered and studied. Among the quality parameters, the most important are represented by nitrate in leaves. The nitrate concentration in leafy vegetables must be below the commercialization limits that are defined by specific EU regulation. At nutritional level, the nitrate intake from food can have negative and positive consequences on human health. Diet rich in nitrate can cause some physiological disorders and the increase of gastrointestinal cancers. For the free commercialization of leafy vegetables, independently form the nitrate effect on human health, limits imposed by the EU regulation n. 1258/2011 (EU 2011) must be respected. The maximum concentrations allowed varying among leafy vegetable species and cultivation period[39]. Nitrate uptake and assimilation are regulated by environmental conditions, since the nitrate reductase activity follows the circadian rhythm and its activity is strictly depended by light intensity and photoperiod[40]. Since the experiment was carried out in autumn-winter period the high nitrate content may be explained by the lower light intensity and temperature. Our results are similar by analogous experiments carried out on lettuce and chicory leafy vegetables. In these vegetables, the nitrate concentration was mainly affected by seasons rather than Se concentration in the nutrient solution[20]. The nitrate leaf accumulation in plant grown under Se treatment did not show any significant effects[21].

However, a reduction of nitrate concentration was found in lettuce treated with 10-120 μmol L^{-1} Se applied as selenite or selenite[41]. In this study, the activation of the main enzymes involved in the nitrogen assimilation such as nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase was observed. In our experiment a decline of nitrate with the increase of Se concentration in the nutrient solution was not observed. The difference of results could be due to the Se application method. In our study Se was directly added in the nutrient solution of the floating system, while in the Rios et al.[41] the Se was distributed with nutrient solution in a pot cultivation. However, in our correlation data a positive and significant correlation was observed. Since nitrate assimilation is affected by light intensity and duration further studies are required. Negative and significant correlations were found between Se and K or Mn. In literature there are no information regarding correlation data between Se and these two elements.

Reducing sugars and sucrose concentrations mainly depend on the crop photosynthesis activity. The concentration of sugars in leaves is important for the storage and shelf life of products. In treatments with Se concentrations the values were higher, but not statistically significant. The increase of sucrose and reducing sugars was also reported in mung [(*Vigna radiata* (L.) R.Wilczek] hydroponically grown with 2.6 or 4.0 µmol L^{-1} (0.5 or 0.75 mg L^{-1}) sodium selenate[42]. Experiments performed in open field on rapeseed (*Brassica napus* L.), Se treatments decreased the reducing sugars concentration. However, Se concentrations applied in rapeseed were comprised from 12.7 to 51 µmol L^{-1} Se[43], higher than those used in our experiments.

Low Se concentrations can also have a plant growth regulator function influencing the antioxidant components, anti-senescence proprieties, abiotic stress regulator, and defensive ability against biotic stresses[44,45].

Conclusion

This study confirms that floating system is an optimal cultivation method for the biofortification of leafy vegetables, since the nutrient solution can be monitored anytime, and mineral composition can be essentially modified by crop uptake. Nutrient solution appropriately prepared can provide the exact amount of the required element in the vegetable that will be part of the consumer's diet. The enriched leafy vegetables can be used in the minimally processed industries for producing commercial bags or package of leafy vegetables with the correct amount of Se that can satisfy the RDA[44]. Our results demonstrated that knowing the accumulation ability of the different species optimized Se concentration in the nutrient solution can be used for producing biofortified vegetables able to satisfy the diet requirements.

The best crop performances were obtained with 2.6 or 3.9 µmol L^{-1} Se concentrations in the nutrient solution, although the four vegetable species used did not show any phytotoxic symptoms even at the highest Se concentration tested. The present work provides original results on the correlation analysis among Se and other mineral elements, suggesting interesting new interactions among them.

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Figure 1. pH (A), EC (B) and temperature (C) of the nutrient solutions in the tanks during cultivation. The values are means (n=3).

Figure 2. Se concentrations in the edible leaves in the different baby leaf vegetables on the fresh (A) or dry (B) weight basis, (C) report the amount of baby leaf required in a daily diet for satisfying the Se RDA (55 µg Se). Values are means with standard errors (n=4). Data in A, B, and C were subjected to two-way ANOVA. Differences among means were determined using Tukey's post-test. Different letters indicate statistical difference for P=0.05.

Figure 3. A) Yield, nitrate B), reducing sugars C), and sucrose D) in lettuce, rocket, spinach, and lamb's lettuce grown in floating system with nutrient solution containing 0, 2.6, 3.9 or 5.2 µmol L-1 Se. Values are means with standard errors (n=3). Data were subjected to two-way ANOVA analysis. Statistical differences among treatments were determined using Tukey's test. Different letters indicate statistical difference for P=0.05.

Figure 4. Biplot of principal component analysis result. Data correspond to rocket (▲), lamb's lettuce (●), lettuce (♦) and spinach (■) plants under Se treatments.

Table 1. Two-way ANOVA data of the different parameters analysed.

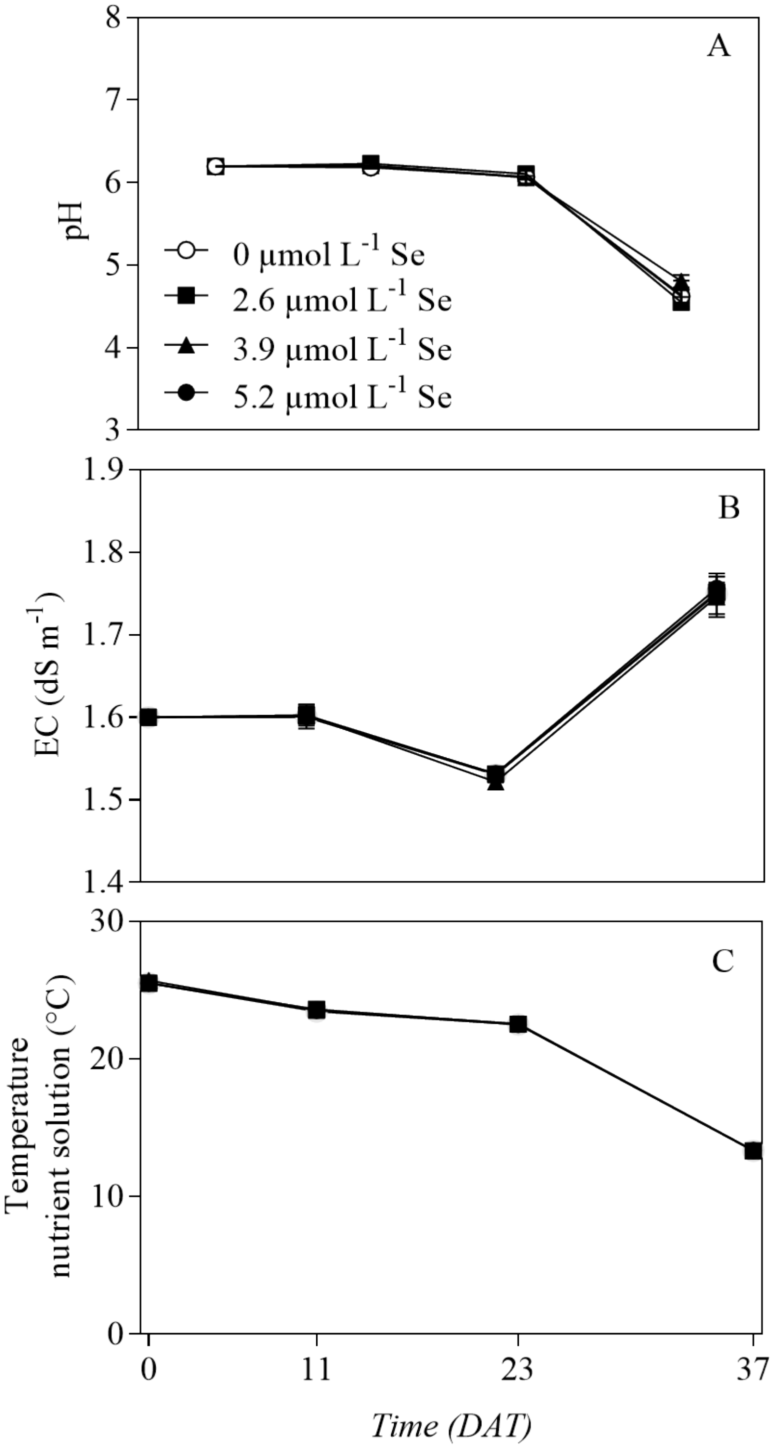
Treatments	**Yield**	**Nitrate**	**Sucrose**	**Reducing sugars**
Se	**(g m^{-2} FW)**	**(mg kg^{-1} FW)**	**(mg g^{-1} FW)**	**(mg g^{-1} FW)**
Control	1409.8	2370.3	0.294	5.850
2.6 µmol L^{-1} Se	1324.3	2123.0	0.719	5.985
3.9 µmol L^{-1} Se	1502.5	2220.9	0.512	6.311
5.2 µmol L^{-1} Se	1349.7	2301.2	0.944	5.836
Species				
Lettuce	1405.3b	2098.7b	0.104c	2.873c
Rocket	1719.7c	3954.0a	0.260b	8.279a
Spinach	440.2d	817.6c	0.110c	7.186a
Lamb's lettuce	2021.2a	2145.1b	1.997a	5.645b
Significance				
Se treatments	0.061 ns	0.702 ns	0.053 ns	0.367 ns
Species	0.0001****	0.0001 ****	0.0001****	0.0001****
Interaction Se *x* Species	0.837 ns	0.835 ns	0.0061 **	0.0001****

ns = not significant; asterisk(s) = significant at 0.005 (**), or 0.0001(****) level of significance.

Table 2. Full correlation matrix between different parameters analysed in all four species of leafy vegetables. Mineral elements, reducing sugars (R. Sug.), sucrose (Sucr.), nitrate, and yield.

	K	**Ca**	**Na**	**Mg**	**B**	**Al**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**	**R. Sug.**	**Sucr.**	**Nitrate**	**Yield**
Se	**-0.391**	**0.675**	**0.397**	0.245	**0.505**	-0.167	-0.102	**-0.402**	-0.131	-0.088	-0.076	-0.014	0.219	-0.121	-0.036	0.247	-0.217	**0.430**	0.147
K		**-0.515**	-0.010	0.344	**-0.499**	-0.099	**-0.288**	**-0.051**	-0.239	-0.267	**-0.291**	**0.416**	0.008	**0.223**	-0.012	**-0.275**	**0.554**	**-0.631**	**-0.620**
Ca			**0.650**	**0.287**	**0.833**	-0.108	0.069	**-0.576**	0.016	0.059	0.003	**0.121**	**0.247**	-0.164	-0.140	0.346	-0.253	**0.795**	0.278
Na				**0.604**	0.335	-0.232	-0.158	**-0.904**	-0.219	-0.214	-0.129	**0.728**	**0.511**	0.099	-0.059	0.095	0.056	**0.367**	-0.293
Mg					0.290	**-0.466**	**-0.476**	**-0.766**	**-0.506**	**-0.473**	**-0.328**	**0.402**	**0.447**	0.084	**-0.388**	**0.344**	**0.532**	-0.131	**-0.767**
B						-0.057	0.088	**-0.321**	0.072	0.101	0.126	-0.129	0.251	-0.190	**-0.185**	**0.457**	**-0.167**	**0.665**	0.233
Al							**0.679**	**0.425**	**0.754**	**0.702**	**0.512**	-0.043	-0.181	0.153	**0.337**	-0.111	-0.231	0.066	**0.333**
Cr								**0.406**	**0.962**	**0.951**	**0.792**	-0.052	-0.280	-0.177	**0.124**	0.006	**-0.303**	**0.261**	**0.484**
Mn									**0.491**	**0.443**	**0.307**	-0.566	**-0.553**	-0.151	**0.173**	**-0.168**	-0.183	**-0.212**	**0.495**
Fe										**0.931**	**0.760**	-0.081	-0.342	-0.178	**0.165**	-0.034	**-0.302**	**0.220**	**0.510**
Co											**0.748**	-0.090	**-0.301**	-0.122	**0.158**	0.021	-0.198	**0.246**	**0.491**
Ni												-0.088	-0.106	-0.085	0.064	0.119	-0.231	**0.132**	**0.297**
Cu													**0.505**	**0.292**	0.174	**-0.207**	**0.201**	0.002	**-0.420**
Zn														**0.359**	0.073	0.058	**0.161**	0.161	**-0.458**
Cd															**0.413**	-0.166	**0.182**	**-0.291**	**-0.265**
Pb																**-0.362**	-0.192	-0.108	0.237
R. Sug.																	0.036	0.210	-0.071
Sucr.																		**-0.398**	**-0.595**
Nitrate																			**0.557**
Yield																			

Coloured *r* coefficients represent statistically significant for $P \leq 0.05$.



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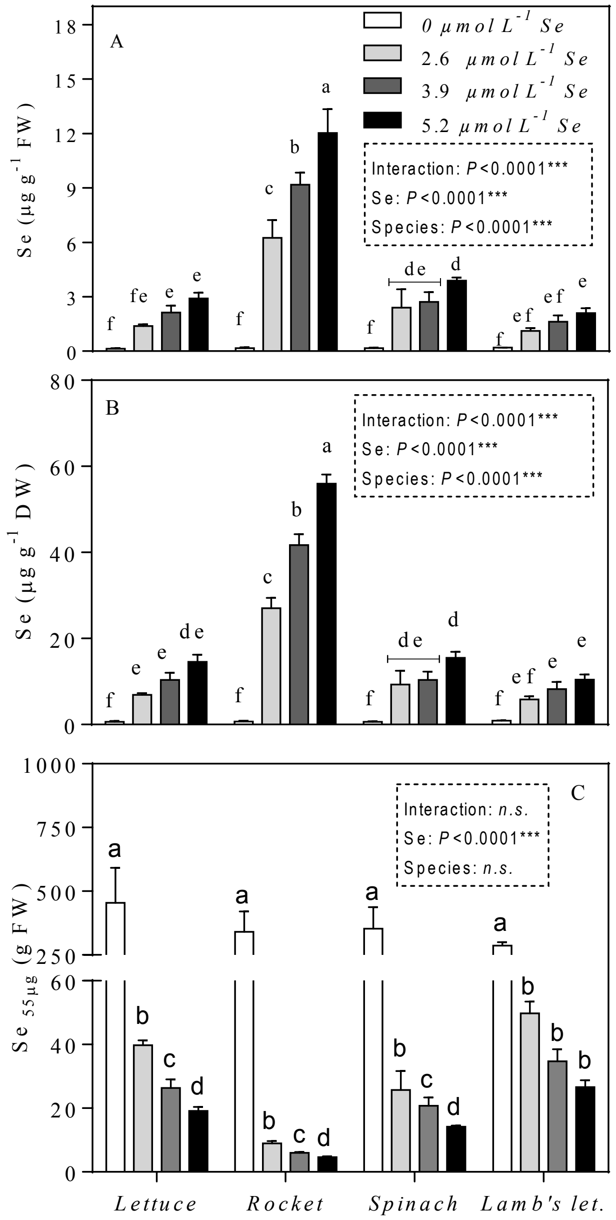

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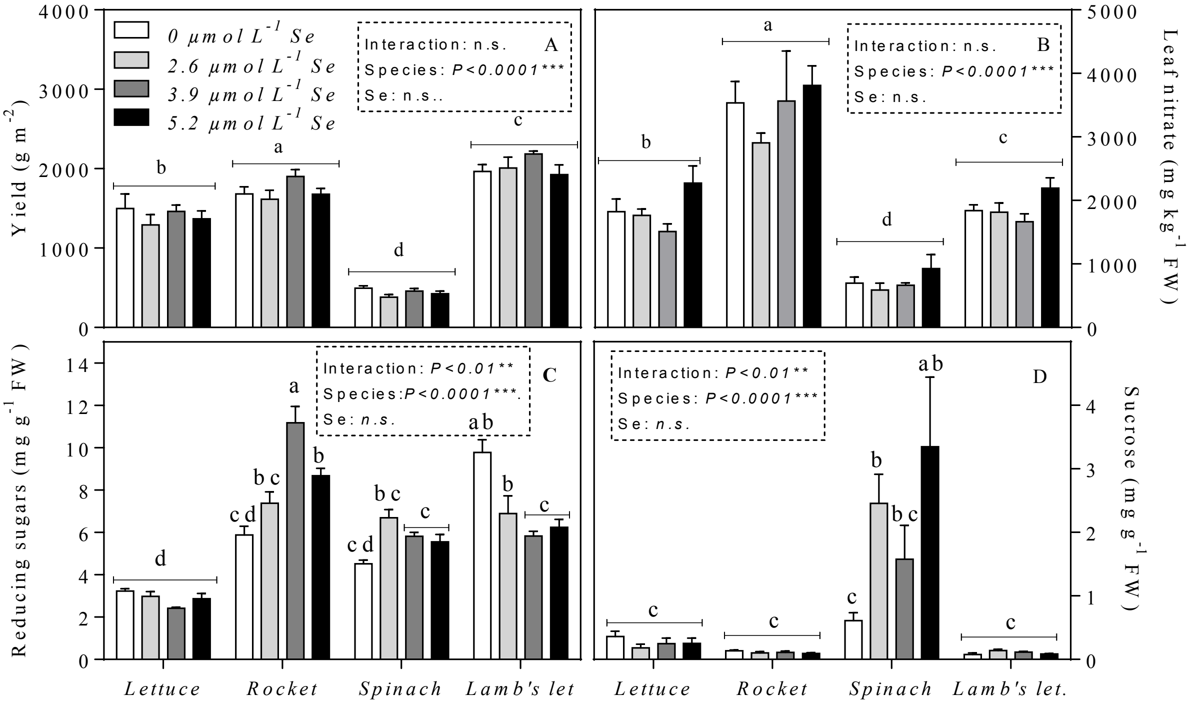

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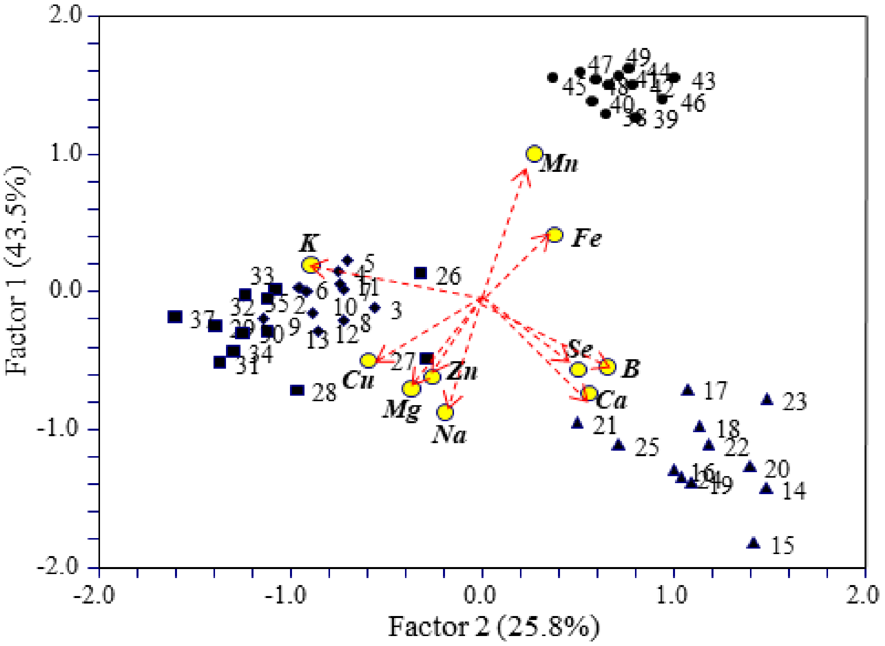

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