

## BIOFORTIFICATION OF SPINACH PLANTS APPLYING SELENIUM IN THE NUTRIENT SOLUTION OF FLOATING SYSTEM

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Received: October 10, 2010; Accepted: April 21, 2012

### Summary

Selenium (Se) is a very important element for human health. It is involved in defense mechanisms and biosynthesis of hormones in adult and babies. The Se is a trace element and in the human body is involved in the membrane protection and has anti-cancer action. The Se is a cofactor of glutathione peroxidase and may play an important role against oxidative tissue damage. The aim of this work was to evaluate the floating system for enriching the baby leaf plants by applying the Se directly in the nutrient solution. This experiment was performed using spinach (*Spinacia oleracea* L.) plants grown in the nutrient solution containing 0, 2.6, 3.9 and 5.2  $\mu\text{M}$  Se applied as  $\text{Na}_2\text{SeO}_4$ .

At harvest time the yield, Se content and quality parameters such as nitrate, reducing sugars and sucrose were determined. The yield was not affected by treatments and depended by seasons.

The Se content in leaves linearly increased with Se concentration in the nutrient solution. The highest value was  $160 \text{ mg}\cdot\text{kg}^{-1}$  DW. Spinach leaves in the 2.6 and 3.9  $\mu\text{M}$  accumulated 9-11  $\mu\text{g}\cdot\text{g}^{-1}$  DW and were the adequate concentrations for providing the recommended dietary allowance of  $55 \mu\text{g}\cdot\text{d}^{-1}$  for adults. Reducing sugars expressed as glucose equivalent did not change among treatments and ranged from 6 to 9  $\text{mg}\cdot\text{g}^{-1}$  FW. The sucrose content ranged from 0.6 to 3  $\text{mg}\cdot\text{g}^{-1}$  FW but no differences were observed among treatments. Nitrate contents in leaves were not affected from Se treatments and ranged from 3898 to  $4475 \text{ mg}\cdot\text{kg}^{-1}$  FW.

key words: fresh-cut, leafy vegetables, minimally processed, ready to eat, sugars, yield

## INTRODUCTION

Vegetables are very important source of antioxidants and nutraceutical compounds for the human diet. The most part of these compounds act against the free radicals/reactive oxygen species (ROS) in plants as well as in humans (Steinbrenner & Sies 2009). ROS include free radicals such as the superoxide anion, hydroxyl, lipid radicals such as oxidizing non-radical species, hydrogen peroxide, peroxy nitrite and singlet oxygen. These deleterious compounds are continuously produced in the respiratory chain of mitochondria by one-electron reduction of molecular oxygen. NAD(P)H oxidases, xanthine oxidase, myeloperoxidase, cyclooxygenase and lipoxygenase are major enzymatic sources of ROS in mammalian cells, but also other abiotic stresses such as UV light exposure that represents an example for an environmental ROS-inducing factor. Selenium (Se) has been demonstrated, as a component of different enzymes such as glutathione peroxidase and thioredoxin reductase, to play an important role in the antioxidant protection of cells (Birringer *et al.* 2002). Se in plant is incorporated in these enzymes as selenocysteine, but there are many others organic forms of Se such as selenomethionine, selenomethylselenocysteine, selenocystathionine, selenomethylselenomethionine, dimethylselenopropionate, dimethylselenide and dimethyldiselenide (Ellis & Salt 2003). It has been clinically demonstrated that Se has also anticarcinogenic properties. However, the optimum concentration of selenium uptake is limited to a very narrow con-

centration range and outside of it deficiency or toxicity may occur.

Biofortification using selenium fertilizers must be performed under very strict conditions, since an over-accumulation in the edible parts might be toxic for consumers. Therefore, the enrichment is usually realized by fertigation avoiding soil supplies that may reach with time toxic levels (Makela *et al.* 1995). High Se concentration in soil may not induce phytotoxic symptoms but reduces the yield of crops.

The hydroponic systems and in particular floating systems represent an easy and closed loop with an optimum control of the mineral concentrations in the nutrient solution. Floating system is one of the most simple cultivation systems composed by a tank containing the nutrient solution and floating panels where the plants are sown and grown (Pardossi *et al.* 2005). The addition of selenium directly in the nutrient solution allows producing enriched leafy vegetables avoiding excessive concentration in the edible parts (Malorgio *et al.* 2009).

The aim of this work was to study the effect of Se concentrations on yield and quality of spinach leaves destined to minimally processed industry. The accumulation ability of spinach, in different Se concentrations, in the nutrient solution and the amount of spinach leaves necessary for satisfying the recommended daily allowance were investigated.

## MATERIALS AND METHODS

### Plant material and growing conditions

Spinach seedlings were grown under plastic greenhouse at the CE.T.A.S. (Centre of Advanced Technologies in Greenhouse, Tavazzano - Lodi) of the University of Milano. Seeds were directly sown in trays (51.5 × 32.5 cm) with 228 holes filled with perlite (density was 1150 seeds·m<sup>-2</sup>). After emergence, the trays were placed in floating tanks (700 L) each containing 4 trays.

The nutrient concentrations (mol·m<sup>-3</sup>) of the feeding solutions, was the 50% of Hoagland's nutrient solution (NS): 6.5 N-NO<sub>3</sub>, 0.75 P, 4 K, 1.75 Ca, 0.85 Mg. The concentration of the micronutrients was (mol·m<sup>-3</sup>): 9.5 Na, 8.0 Cl, 2.7 S, 0.04 Fe while other micronutrients were (μmol·m<sup>-3</sup>): 9 B, 0.1 Co, 3 Cu, 15 Mn, 0.1 Mo, 10 Zn. The EC at beginning of experiment was 1.5 to 1.6 and pH was adjusted to 6.0 with 77 mL sulfuric acid.

Four cultivation tanks were placed in randomized blocks in greenhouse and in each tank there were four floating panels. The leaves were harvested at commercial stage, when plants reached the 10-15 cm height. Temperature and solar radiation outside greenhouse were monitoring during the whole experimental period (Fig. 1).

### Yield, Nitrate content, sucrose and reducing sugars determination

Yield was determined at harvest time. Nitrate content was measured by spectrophotometry using the salicylic-sulphuric acid method. For each sample 100 mg of DW was ground and placed in 30 ml icon glass with 10 ml

of distilled water. Samples were placed on a shaking machine for 2 h at room temperature. After shaking, 10 ml of samples were centrifuged at 4000 rpm for 15 min. The supernatant was recovered and 200 μl were added to 800 μl of 5% salicylic acid in sulphuric acid. The icon glass was placed on the stirring machine and 30 ml of 1.5 mol·L<sup>-1</sup> NaOH were slowly added. The samples were usually cooled to room temperature and spectrophotometer readings were performed at 410 nm (Cataldo *et al.* 1975). The nitrate content was calculated using calibration standards containing 0, 1, 2.5, 5, 7.5 and 10 mmol·L<sup>-1</sup> KNO<sub>3</sub>.

For the determination of sucrose and reducing sugars, 2 g of tissue were extracted by homogenization in a mortar with water or 0.1% TCA as buffers in order to reduce the analytical assay procedures. The insoluble material was separated by centrifugation at 10000 rpm for 5 min.

The sucrose assay was performed by mixing 0.2 mL of crude extract with 0.2 mL NaOH 2N and incubated in a water bath at 100°C for 10 min., then 1.5 ml of hot resorcinol buffer was added to samples and incubated in a water bath at 80°C for other 10 min. Resorcinol solution was prepared by mixing 250 ml HCl 30%, 35 mg resorcinol (Sigma, Italy), 90 mg thiourea (Sigma, Italy), 25 ml acetic acid and 10 ml distilled water. After cooling at room temperature, the optical density was determined spectrophotometrically at 500 nm, using a sucrose standard curve (0, 0.5, 1, 1.5 and 2 mM).

The reducing sugars determinations were performed using 0.2 mL of crude extract that were added to 0.2

mL of dinitrosalicylic acid (DNS) the reaction mixture was heated at 100 °C for 5 min, then 1.5 mL of distilled water was added and absorbance readings were taken at 530 nm. The reducing sugars were expressed as glucose equivalent using a standard curve (0, 1, 2, 3 and 4 mM).

#### Se determination

Dry materials were ground and digested with nitric acid. Se was measured using inductively coupled plasma mass spectrometry (ICP-MS).

#### Statistical analyses

The data reported are means with standard errors ( $n=4$ ). The significance of the effect of nutrient concentration was assessed by one-way ANOVA and the differences among treatments were analyzed by Tukey's multiple comparison test ( $P<0.05$ ).

## RESULTS

#### The pH and the EC of nutrient solutions

During cultivation the pH lowered in all treatments and declined until 4.5-4.8 at the end of growing cycle (Table

1). The EC was stable during the first 23 days of cultivation than increased up to reach 1.7-1.8  $\text{ms}\cdot\text{cm}^{-1}$ .

#### Yield, nitrate, sucrose and reducing sugars

Se treatments did not significantly affect the parameters measured. The yield of spinach ranged between 381 and 495  $\text{g}\cdot\text{m}^{-2}$ , the highest value was found in control. The growing cycle was 27 days long. Nitrate content was very high in all treatments since overcome the commercialization limits imposed by EU regulation. Values ranged between 3552 and 4475  $\text{mg}\cdot\text{kg}^{-1}$  FW. The higher values were observed in control and 5.2  $\mu\text{M}$  Se. Differences were not significant among treatments. The sucrose content in spinach leaves increased by increasing the Se concentration in the nutrient solution of floating system (Table 2). The reducing sugars were higher in leaves of plants grown in nutrient solution containing 2.6 and 3.9  $\mu\text{M}$  of Se. The reducing sugars values ranged from 4.5 to 6.2  $\text{mg}\cdot\text{g}^{-1}$  FW but differences were not statistically different among treatments.

Table 1. The pH and the EC of nutrient solutions in the different Se treatments during the cultivation period. The values are means ( $n=3$ ).

Time (d)	pH				EC (mS/cm)			
	Se ( $\mu\text{M}$ ) treatments				Se ( $\mu\text{M}$ ) treatments			
	0	2.6	3.9	5.2	0	2.6	3.9	5.2
11	6.2	6.2	6.2	6.2	1.6	1.6	1.6	1.6
23	6.1	6.1	6.1	6.1	1.5	1.5	1.5	1.5
37	4.6	4.5	4.8	4.6	1.7	1.7	1.7	1.8

Table 2. Yield, nitrate, sucrose and reducing sugar content in spinach leaves grown in floating system with nutrient solution containing 0, 2.6, 3.9 or 5.2  $\mu\text{M}$  Se. Values are means with standard errors (n=3). Data were subjected to one-way ANOVA analysis. Statistical differences among treatments were determined using Tukey's test

Se $\mu\text{M}$	Yield $\text{g}\cdot\text{m}^{-2}$	Nitrates $\text{mg}\cdot\text{kg}^{-1}$ FW	Sucrose $\text{mg}\cdot\text{g}^{-1}$ FW	Reducing sugars $\text{mg}\cdot\text{g}^{-1}$ FW
0	495.3 $\pm$ 29.72	4475 $\pm$ 503.3	0.62 $\pm$ 0.220	4.54 $\pm$ 0.699
2.6	381.3 $\pm$ 34.26	3552 $\pm$ 394.5	2.46 $\pm$ 0.796	6.19 $\pm$ 0.632
3.9	458.7 $\pm$ 34.34	3898 $\pm$ 267.6	1.58 $\pm$ 0.924	6.09 $\pm$ 0.676
5.2	425.3 $\pm$ 34.26	4258 $\pm$ 691.6	3.35 $\pm$ 1.901	5.44 $\pm$ 0.637
<i>P</i>	0.174	0.584	0.414	0.333
Significant level	ns	ns	ns	ns

### Selenium content in spinach leaves

The Se content in dry matter increased with Se concentration in the nutrient solution (Fig. 2A). Plants grown in the intermediate Se concentrations did not show significant differences in accumulation and values ranged between 9.3 and 10.3  $\mu\text{g}\cdot\text{g}^{-1}$  DW. Spinach plants grown with the highest Se concentration in the nutrient solution showed the highest content with a mean of 15.5  $\mu\text{g}\cdot\text{g}^{-1}$  DW.

Since the leafy vegetables are eaten as fresh product the Se concentration

was also expressed on the fresh weight basis. Results did not show significant differences among treatments and values ranged from 2.4 to 3.9  $\mu\text{g}\cdot\text{g}^{-1}$  FW while in the control the Se content was in average 0.16  $\mu\text{g}\cdot\text{g}^{-1}$  FW (Fig. 2B).

The recommended dietary allowance (RDA) for Se is 55  $\mu\text{g}\cdot\text{day}^{-1}$  for adults, therefore the amount of spinach that should be eaten for satisfy the RDA has been calculated (Fig. 2C). The Se 2.6, 3.9 and 5.2  $\mu\text{M}$  Se provide the RDA with 25.7, 20.8 and 14.2 g respectively (Fig. 2C).

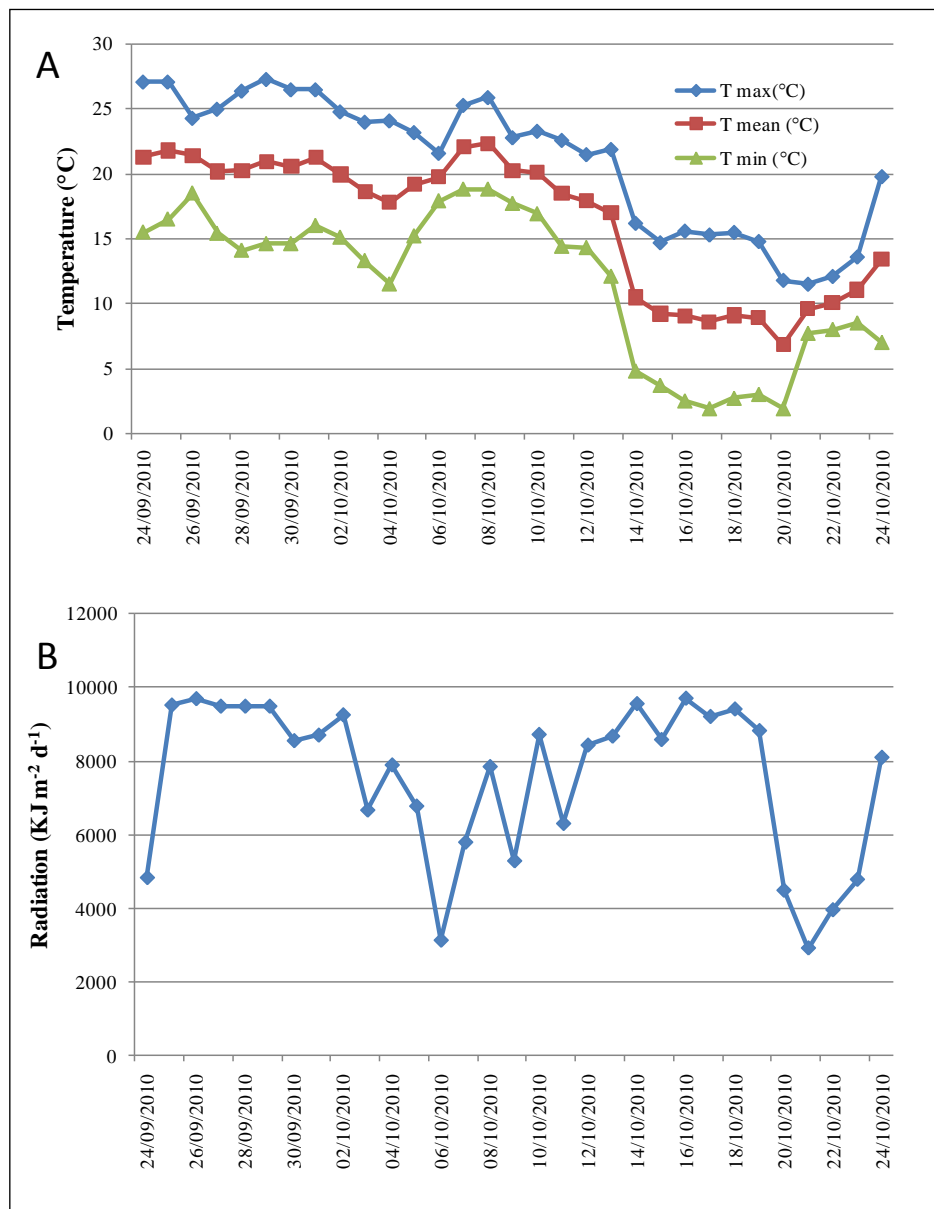


Fig. 1. Outside greenhouse temperatures and solar radiation during the cultivation period

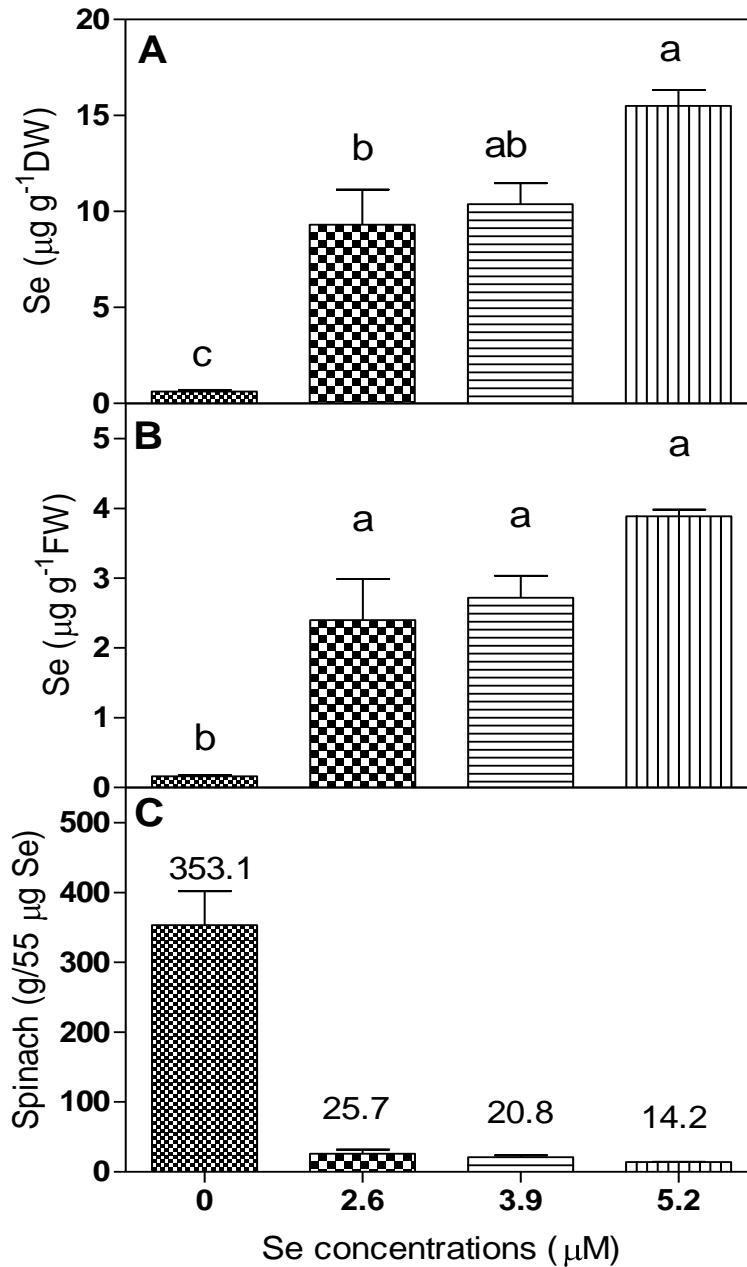


Fig. 2. Selenium (Se) content expressed on DW (A) or FW (B) basis in spinach leaves grown in floating system with nutrient solution containing 0, 2.6, 3.9 or 5.2 μM Se. The amount of spinach that provides 55 μg day<sup>-1</sup> (C). Values are means with standard errors (n=3). Data were subjected to one-way ANOVA analysis. Statistical differences among treatments were determined using Tukey's test. Different letters mean statistical differences for  $p \leq 0.05$ .

## DISCUSSION

The biofortification of vegetables with selenium must be performed considering the toxicity of this element for plants and humans. In open field crop enrichment is achieved by fertilizers applied as foliar spraying (Germ *et al.* 2007). Closed hydroponic systems, such as floating system, allow controlling the uptake and residual concentration in the nutrient solution.

In our work the yield was not affected by Se treatments. Contrary results have been reported for lettuce and chicory leafy vegetables that showed a significant increase of yield (Malorgio *et al.* 2009). However, the environmental conditions and seasons strongly affect the yield in spinach (Alberici *et al.* 2008). Nitrate content in leaves is also regulated by environmental conditions since the nitrate reductase activity follows the circadian rhythm and its activity is strictly depended by light intensity and duration (Antonacci *et al.* 2007). The experiment was performed in autumn-winter period with adverse environmental conditions (Fig. 1) and may explain the high nitrate content.

It seems that is not related with Se concentration in the growing media. These results are also confirmed by experiments performed on chicory and lettuce leafy vegetables. In these species the nitrate content was mainly affected by seasons rather than Se concentration in the nutrient solution (Malorgio *et al.* 2009).

Sucrose and reducing sugars content mainly depend by photosynthesis activity. In intermediate Se concentration the values were higher but the wide variability resulted in no

statistically differences. In experiments conducted in open field on rapeseed (*Brassica napus* L.) showed that Se treatments decreased the reducing sugars content. However the concentrations used in rapeseed were much higher, ranging from 1 to 4 mg Se·kg<sup>-1</sup> (Sharma *et al.* 2010), compared to those applied in our experiments.

Spinach Se content obtained with the highest concentration was similar to that found in lettuce and chicory grown with 0.5 mg Se·L<sup>-1</sup> (Malorgio *et al.* 2009). Analogous Se accumulations were observed in the edible parts of cabbage, radish, garlic and onion supplied with 20 mg·m<sup>-2</sup> Se (Slekovec & Goessler 2005).

In human diet the consumption of food with less than 0.1 mg Se·kg<sup>-1</sup> results in deficiency, while the intake of food with Se content higher than 1 g S·kg<sup>-1</sup> induces toxicity (Whanger 2002). The floating system is an optimum method for enriching leafy vegetables because the Se can be constantly monitored during and after the cultivation cycle, avoiding excessive accumulation in plants. However, the leafy vegetables grown in the higher Se concentrations can be used for making mix salads. Therefore the correct mixing can provide in each single bag the RDA to consumers at each meal.

In conclusion, this work suggests that leafy vegetables can be enriched with Se in floating system providing a good concentration in the product that can be used alone or in mixed salads. Spinach did not show any phytotoxic symptoms but the best performances were obtained with intermediate Se concentrations.



### Acknowledgements

The present work was funded by Regional Government of Lombardia (Project SOL09 "Biofortification of leafy vegetables grown in floating system – Rete ColtureOrticole").

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## BIO-WZMACNIANIE ROŚLIN SZPINAKU SELENEM STOSOWANYM W ROZTWORZE ODŻYWCZYM W UPRAWIE HYDROPONICZNEJ

### Streszczenie

Selen (Se) jest bardzo ważnym pierwiastkiem dla zdrowia ludzkiego. Bierze on udział w mechanizmach obronnych i biosyntezie hormonów u dorosłych i dzieci. Selen jest pierwiastkiem śladowym. W organizmie człowieka uczestniczy w ochronie membrany komórkowej i ma działanie przeciwnowotworowe. Selen jest kofaktorem peroksydazy glutationowej i może odgrywać istotną rolę w ochronie przed uszkodzeniem oksydacyjnym tkanki. Celem niniejszej pracy była ocena wzbogacania młodych roślin liściastych, poprzez zastosowanie selenu bezpośrednio w roztworze odżywczym. Doświadczenie to przeprowadzono wykorzystując rośliny szpinaku (*Spinacia oleracea* L.), rosące w pożywce płynnej zawierającej 0; 2,6; 3,9; i 5,2  $\mu\text{M}$  Se, zastosowanego w formie  $\text{Na}_2\text{SeO}_4$ .

W okresie zbiorów określano plon, zawartość selenu i parametry jakościowe, takie jak: zawartość azotanów, cukrów redukujących oraz sacharozy. Traktowanie roślin selenem nie miało wpływu na wysokość plonu; zależał on od terminu uprawy.

Zawartość selenu w liściach wzrastała liniowo wraz ze stężeniem Se w pożywce. Najwyższa wartość wynosiła  $160 \text{ mg}\cdot\text{kg}^{-1}$  s.m. Przy zawartości Se w pożywce 2,6 i 3,9  $\mu\text{M}$  liście szpinaku nagromadziły  $9\text{-}11 \mu\text{g}\cdot\text{g}^{-1}$  s.m. i były to odpowiednie stężenia do zapewnienia zalecanego limitu żywieniowego, wynoszącego dla dorosłych  $55 \mu\text{g}\cdot\text{d}^{-1}$ . Zawartość cukrów redukujących, wyrażonych jako ekwiwalent glukozy, nie zmieniała się istotnie i wahała się od 6 do  $9 \text{ mg}\cdot\text{g}^{-1}$  św.m. Zawartość sacharozy wynosiła od 0,6 do  $3 \text{ mg}\cdot\text{g}^{-1}$  św.m., ale nie stwierdzono różnic istotnych pomiędzy kombinacjami. Zawartość azotanów w liściach nie zależała od zawartości selenu i wahała się od 3898 do  $4475 \text{ mg}\cdot\text{kg}^{-1}$  św.m.